

**RANDOM PEPTIDES THAT BIND TO GASTRO-INTESTINAL  
TRACT (GIT) TRANSPORT RECEPTORS AND RELATED METHODS**

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This application claims priority to U.S.  
provisional application Serial No. 60/046,595 filed May 15,  
1997, which is incorporated by reference herein in its  
entirety.

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**1. INTRODUCTION**

The present invention relates generally to random  
peptides capable of specific binding to gastro-intestinal  
tract (GIT) transport receptors. In particular, this  
15 invention relates to peptide sequences and motifs, as well as  
derivatives thereof, which enhance drug delivery and  
transport through tissue, such as epithelial cells lining the  
luminal side of the gastro-intestinal tract (GIT).  
Production of peptides, derivatives and antibodies is also  
20 provided. The invention further relates to pharmaceutical  
compositions, formulations and related methods.

**2. BACKGROUND OF THE INVENTION**

**2.1. Peptide Libraries**

25 There have been two different approaches to the  
construction of random peptide libraries. According to one  
approach, peptides have been chemically synthesized *in vitro*  
in several formats. Examples of chemically synthesized  
libraries can be found in Fodor, S., et al., 1991, Science  
30 251: 767-773; Houghten, R., et al., 1991, Nature 354: 84-86;  
and Lam, K., et al., 1991, Nature 354: 82-84.

A second approach to the construction of random  
peptide libraries has been to use the M13 phage, and, in  
particular, protein pIII of M13. The viral capsid protein of  
35 M13, protein III (pIII), is responsible for infection of  
bacteria. Several investigators have determined from  
mutational analysis that the 406 amino acid long pIII capsid

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protein has two domains. The C-terminus anchors the protein to the viral coat, while portions of the N-terminus of pIII are essential for interaction with the *E. coli* pillin protein (Crissman, J.W. and Smith, G.P., 1984, Virology 132: 445-  
5 455). Although the N-terminus of the pIII protein has shown to be necessary for viral infection, the extreme N-terminus of the mature protein does tolerate alterations. In 1985, George Smith published experiments reporting the use of the pIII protein of bacteriophage M13 as an experimental system  
10 for expressing a heterologous protein on the viral coat surface (Smith, G.P., 1985, Science 228: 1315-1317). It was later recognized, independently by two groups, that the M13 phage pIII gene display system could be a useful one for mapping antibody epitopes (De la Cruz, V., et al., 1988,  
15 J. Biol. Chem. 263: 4318-4322; Parmley, S.F. and Smith, G.P., 1988, Gene 73: 305-318).

Parmley, S.F. and Smith, G.P., 1989, Adv. Exp. Med. Biol. 251: 215-218 suggested that short, synthetic DNA segments cloned into the pIII gene might represent a library  
20 of epitopes. These authors reasoned that since linear epitopes were often ~6 amino acids in length, it should be possible to use a random recombinant DNA library to express all possible hexapeptides to isolate epitopes that bind to antibodies. Scott, J.K. and Smith, G.P., 1990, Science 249:  
25 386-390 describe construction and expression of an "epitope library" of hexapeptides on the surface of M13. Cwirla, S.E., et al., 1990, Proc. Natl. Acad. Sci. USA 87: 6378-6382 also described a somewhat similar library of hexapeptides expressed as gene pIII fusions of M13 fd phage. PCT  
30 Application WO 91/19818 published December 26, 1991 by Dower and Cwirla describes a similar library of pentameric to octameric random amino acid sequences. Devlin et al., 1990, Science, 249: 404-406, describes a peptide library of about 15 residues generated using an (NNS) coding scheme for  
35 oligonucleotide synthesis in which S is G or C. Christian and colleagues have described a phage display library,

expressing decapeptides (Christian, R.B., et al., 1992, J. Mol. Biol. 227: 711-718).

Other investigators have used other viral capsid proteins for expression of non-viral DNA on the surface of phage particles. For example, the major capsid protein pVIII was so used by Cesareni, G., 1992, FEBS Lett. 307: 66-70. Other bacteriophage than M13 have been used to construct peptide libraries. Four and six amino acid sequences corresponding to different segments of the Plasmodium falciparum major surface antigen have been cloned and expressed in the filamentous bacteriophage fd (Greenwood, J., et al., 1991, J. Mol. Biol. 220: 821-827).

Kay et al., 1993, Gene 128: 59-65 (Kay) discloses a method of constructing peptide libraries that encode peptides of totally random sequence that are longer than those of any prior conventional libraries. The libraries disclosed in Kay encode totally synthetic random peptides of greater than about 20 amino acids in length. Such libraries can be advantageously screened to identify peptides, polypeptides and/or other proteins having binding specificity for a variety of ligands. (See also U.S. Patent No. 5,498,538 dated March 12, 1996; and PCT Publication No. WO 94/18318 dated August 18, 1994.)

A comprehensive review of various types of peptide libraries can be found in Gallop et al., 1994, J. Med. Chem. 37:1233-1251.

Screening of peptide libraries has often been done using an antibody as ligand (Parmley and Smith, 1989, Adv. Exp. Med. Biol. 251:215-218; Scott and Smith, 1990, Science 249:386-390). In many cases, the aim of the screening is to identify peptides from the library that mimic the epitopes to which the antibodies are directed. Thus, given an available antibody, peptide libraries are excellent sources for identifying epitopes or epitope-like molecules of that antibody (Yayon et al., 1993, Proc. Natl. Acad. Sci. USA 90:10643-10647).

McCafferty et al., 1990, Nature 348:552-554 used PCR to amplify immunoglobulin variable (V) region genes and cloned those genes into phage expression vectors. The authors suggested that phage libraries of V, diversity (D), and joining (J) regions could be screened with antigen. The phage that bound to antigen could then be mutated in the antigen-binding loops of the antibody genes and rescreened. The process could be repeated several times, ultimately giving rise to phage which bind the antigen strongly.

10 Marks et al., 1991, J. Mol. Biol. 222:581-597 also used PCR to amplify immunoglobulin variable (V) region genes and cloned those genes into phage expression vectors.

Kang et al., 1991, Proc. Natl. Acad. Sci. USA 88:4363-4366 created a phagemid vector that could be used to express the V and constant (C) regions of the heavy and light chains of an antibody specific for an antigen. The heavy and light chain V-C regions were engineered to combine in the periplasm to produce an antibody-like molecule with a functional antigen binding site. Infection of cells harboring this phagemid with helper phage resulted in the incorporation of the antibody-like molecule on the surface of phage that carried the phagemid DNA. This allowed for identification and enrichment of these phage by screening with the antigen. It was suggested that the enriched phage could be subject to mutation and further rounds of screening, leading to the isolation of antibody-like molecules that were capable of even stronger binding to the antigen.

Hoogenboom et al., 1991, Nucleic Acids Res. 19:4133-4137 suggested that naive antibody genes might be cloned into phage display libraries. This would be followed by random mutation of the cloned antibody genes to generate high affinity variants.

Bass et al., 1990, Proteins: Struct. Func. Genet. 8:309-314 fused human growth hormone (hGH) to the carboxy terminus of the gene III protein of phage fd. This fusion protein was built into a phagemid vector. When cells carrying the phagemid were infected with a helper phage,

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about 10% of the phage particles produced displayed the fusion protein on their surfaces. These phage particles were enriched by screening with hGH receptor-coated beads. It was suggested that this system could be used to develop mutants  
5 of hGH with altered receptor binding characteristics.

Lowman et al., 1991, Biochemistry 30:10832-10838 used an improved version of the system of Bass et al. described above to select for mutant hGH proteins with exceptionally high affinity for the hGH receptor. The  
10 authors randomly mutagenized the hGH-pIII fusion proteins at sites near the vicinity of 12 amino acids of hGH that had previously been identified as being important in receptor binding.

Balass et al., 1993, Proc. Natl. Acad. Sci. USA  
15 90:10638-10642 used a phage display library to isolate linear peptides that mimicked a conformationally dependent epitope of the nicotinic acetylcholine receptor. This was done by screening the library with a monoclonal antibody specific for the conformationally dependent epitope. The monoclonal  
20 antibody used was thought to be specific to the acetylcholine receptor's binding site for its natural ligand, acetylcholine.

## 2.2. Drug Delivery Systems

25 The common routes of therapeutic drug administration are oral ingestion or parenteral (intravenous, subcutaneous and intramuscular) routes of administration. Intravenous drug administration suffers from numerous limitations, including (i) the risk of adverse effects  
30 resulting from rapid accumulation of high concentrations of drug, (ii) repeated injections which can cause patient discomfort; and (iii) the risk of infection at the site of repeated injections. Subcutaneous injection is not generally suitable for delivering large volumes or for irritating  
35 substances. Whereas oral administration is generally more convenient, it is limited where the therapeutic agent is not efficiently absorbed by the gastrointestinal tract. To date,

the development of oral formulations for the effective delivery of peptides, proteins and macromolecules has been an elusive target. Poor membrane permeability, enzymatic instability, large molecular size, and hydrophilic properties are four factors that have remained major hurdles for peptide and protein formulations (reviewed by Fix, J.A., 1996, J. Pharmac. Sci. 85:1282-1285). In order to develop an efficacious oral formulation, the peptide must be protected from the enzymatic environment of the gastrointestinal tract (GIT), presented to the absorptive epithelial barrier in a sufficient concentration to effect transcellular flux (Fix, J.A., 1996, J. Pharmac. Sci. 85:1282-1285), and if possible "smuggled" across the epithelial barrier in an apical to basolateral direction.

Site specific drug delivery or drug targeting can be achieved at different levels, including (i) primary targeting to a specific organ, (ii) secondary targeting to a specific cell type within that organ and (iii) tertiary targeting where the drug is delivered to specific intracellular structures (e.g., the nucleus for genes) (reviewed in Davis and Jllum, 1994, In: Targeting of Drugs 4, (Eds), Gregoriadis, McCormack and Poste, 183-194). At present there is a considerable amount of ongoing research work in the Drug Delivery Systems (DDS) area, and much of it addresses (i) targeting delivery and (ii) the development of non-invasive ways of getting macromolecules, peptides, proteins, products of the biotechnology industry, etc. into the body (Evers, P., 1995, Developments in Drug Delivery: Technology and Markets, Financial Times Management Report).

It is generally accepted that targeted drug delivery is crucial to the improved treatment of certain diseases, especially cancer, and not surprisingly many of the approaches to targeted drug delivery are focused in the cancer area. Many anticancer drugs are toxic to the body as well as to malignant cells. If a drug, or a delivery system, can be modified so that it "homes in" on the tumor, then by maximizing the drug concentration at the disease site, the

anti-cancer effect can be exploited to the full, while toxicity is greatly reduced. Tumors contain antigens which provoke the body to respond by producing antibodies designed to attach to the antigens and destroy them. Monoclonal  
5 antibodies are being used as both delivery vehicles targeted to tumor cells (reviewed by Pietersz, G.A., 1990, Bioconjugate Chem. 1:89-95) and as imaging agents to carry molecules of drug or imaging agent to the tumor surface.

10        2.3. Transport Pathways

The epithelial cells lining the luminal side of the GIT are a major barrier to drug delivery following oral administration. However, there are four recognized transport pathways which can be exploited to facilitate drug delivery  
15 and transport: the transcellular, paracellular, carrier-mediated, and transcytotic pathways. The ability of a conventional drug, peptide, protein, macromolecule or nano- or microparticulate system to "interact" with one of these transport pathways may result in increased delivery of that  
20 drug or particle from the GIT to the underlying circulation.

In the case of the receptor-mediated, carrier-mediated or transcytotic transport pathways, some of the uptake signals have been identified. These signals include, *inter alia*, folic acid, which interacts with the folate  
25 receptor, and cobalamin, which interacts with Intrinsic Factor. In addition, leucine- and tyrosine-based peptide sorting motifs or internalization sequences exist, such as YSKV, FPHL, YRGV, YQTI, TEQF, TEVM, TSAF, and YTRF (SEQ ID NOS:203, 204, 205, 206, 207, 208, 209, and 210,  
30 respectively), which facilitate uptake or targeting of proteins using specific membrane receptors or binding sites to identify peptides that bind specifically to the receptor or binding site.

Non-receptor based assays to discover particular  
35 ligands have also been used. For instance, a strategy for identifying peptides that alter cellular function by scanning whole cells with phage display libraries is disclosed in Fong

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et al., Drug Development Research 33:64-70 (1994). However, because whole cells, rather than intact tissue or polarized cell cultures, are used for screening phage display libraries, this procedure does not provide information  
5 regarding sequences whose primary function includes affecting transport across polarized cell layers.

Additionally, Stevenson et al., Pharmaceutical Res. 12(9), S94 (1995) discloses the use of Caco-2 monolayers to screen a synthetic tripeptide combinatorial library for  
10 information relating to the permeability of di- and tri-peptides.

A method of identifying a peptide which permits or facilitates the transport of an active agent through human or animal tissues has been developed (see U.S. patent  
15 application Serial No. 08/746,411 filed November 8, 1996, which is incorporated by reference herein in its entirety). Phage from a random phage library is plated onto or brought into contact with a first side, preferably the apical side, of a tissue sample, either *in vitro*, *in vivo* or *in situ*, or  
20 polarized tissue cell culture. The phage which is transported to a second side of the tissue opposite the first side, preferably the basolateral side, is harvested to select transported phages. The transported phages are amplified in a host and this cycle is repeated (using the transported  
25 phage from the most recent cycle) to obtain a selected phage library containing phage which can be transported from the first side to the second side.

Discussion or citation of a reference hereinabove shall not be construed as meaning that such reference is  
30 prior art to the present invention.

### 3. SUMMARY OF THE INVENTION

The present invention relates generally to random peptides and peptide motifs capable of specific binding to  
35 GIT transport receptors. Such proteins can be identified using any random peptide library, e.g., a chemically synthesized peptide library or a biologically expressed

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peptide library. If a biological peptide expression library is used, the nucleic acid which encodes the peptide which binds to the ligand of choice can be recovered, and then sequenced to determine its nucleotide sequence and hence  
5 deduce the amino acid sequence that mediates binding. Alternatively, the amino acid sequence of an appropriate binding domain can be determined by direct determination of the amino acid sequence of a peptide selected from a peptide library containing chemically synthesized peptides. In a  
10 less preferred aspect, direct amino acid sequencing of a binding peptide selected from a biological peptide expression library can also be performed.

In particular, this invention relates to proteins (e.g., peptides) that are capable of facilitating transport  
15 of an active agent through a human or animal gastrointestinal tissue, and derivatives (e.g., fragments) and analogs thereof, and nucleotide sequences coding for said proteins and derivatives.

Preferably, the tissue through which transport is  
20 facilitated is of the duodenum, jejunum, ileum, ascending colon, transverse colon, descending colon, or pelvic colon. The tissue is most preferably epithelial cells lining the luminal side of the GIT.

The proteins of the invention have use in  
25 facilitating transport of active agents from the luminal side of the GIT into the systemic blood system, and/or in targeting active agents to the GIT. Thus, for example, by binding (covalently or noncovalently) a protein of the invention to an orally administered drug, the drug can be  
30 targeted to specific receptor sites or transport pathways which are known to operate in the human gastrointestinal tract, thus facilitating its absorption into the systemic system.

The invention also relates to derivatives and  
35 analogs of the invention which are functionally active, i.e., they are capable of displaying one or more known functional activities associated with a full-length peptide. Such

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functional activities include but are not limited to antigenicity (ability to bind or to compete with GIT transport receptor-binding peptides for binding to an anti-GIT transport receptor antibody) and ability to bind or  
5 compete with full-length peptide for binding to a GIT transport receptor.

The invention further relates to fragments of (and derivatives and analogs thereof) GIT transport receptor-binding peptides which comprise one or more motifs of a GIT  
10 transport receptor-binding peptide.

Antibodies to GIT transport receptor-binding peptides and GIT transport receptor-binding peptide derivatives and analogs are additionally provided.

Methods of production of the GIT transport  
15 receptor-binding peptides, derivatives, fragments and analogs, e.g., by recombinant means, are also provided.

The present invention also relates to therapeutic methods, pharmaceutical compositions and formulations based on GIT transport receptor-binding peptides. Formulations of  
20 the invention include but are not limited to GIT transport receptor-binding peptides or motifs and derivatives (including fragments) thereof; antibodies thereto; and nucleic acids encoding the GIT transport receptor-binding peptides or derivatives associated with an active agent.  
25 Preferably, the active agent is a drug or drug-containing nano- or microparticle.

The GIT transport-receptor binding proteins of the invention can also be used to determine levels of the GIT transport receptors in a sample by binding thereto.

30 The GIT transport-receptor binding proteins can also be used to identify molecules that bind thereto, by contacting candidate test molecules under conditions conducive to binding, and detecting any binding that occurs.

#### 35 4. DESCRIPTION OF THE FIGURES

**Figure 1.** Figure 1 shows the human PEPT1 predicted amino acid sequence determined from the sequence of the cDNA clone

coding for human PEPT1 (SEQ ID NO:176) (Liang R. et al. J. Biol. Chem. 270(12):6456-6463 (1995)), including the extracellular domain from amino acid 391 to 573 (Fei et al., Nature 368:563 (1994)).

**5 Figures 2A-2C.** Figures 2A-2C show the DNA sequence of the cDNA coding for the human intestinal peptide-associated transporter HPT1 and the corresponding putative amino acid sequence (bases 1 to 3345; Medline:94204643) (SEQ ID NOS: 177 and 178, respectively).

**10 Figures 3A-3B.** Figures 3A-3B show the putative Human Sucrase-isomaltase complex(hSI) amino acid sequence determined from the sequence of the cDNA clone coding for human sucrase-isomaltase complex (SEQ ID NO:179) (Chantret I., et al., Biochem. J. 285(Pt 3):915-923 (1992)).

**15 Figures 4A-4B.** Figures 4A-4B show the D2H nucleotide and deduced amino acid sequence for the human D2H transporter (SEQ ID NOS:180 and 181, respectively) (Wells, R.G. et al., J. Clin. Invest. 90:1959-1963 (1993)).

**Figures 5A-5C.** Figure 5A is a schematic summary of the  
**20** cloning of the DNA insert present in gene III of the phages selected from the phage display libraries into the expression vector pGex-4T-2. The gene insert in gene III of the phages was amplified by PCR using DNA primers which flank the gene insert and which contained recognition sequences for specific  
**25** restriction endonucleases at their extreme 5' sides.

Alternatively, specific primers which amplify specific regions of the DNA inserts in gene III of the phages, and which contained recognition sequences for specific restriction endonucleases at their extreme 5' sides, were  
**30** used in PCR amplification experiments. Following amplification of the gene inserts, the amplified PCR fragments were digested with the restriction endonucleases Xho1 and Not1. Similarly the plasmid pGex-4T-2, which codes for the reporter protein glutathione S-transferase (GST), was  
**35** digested with the restriction endonucleases Sal1 and Not1. The digested PCR fragments were ligated into the digested plasmid pGex-4T-2 using T4 DNA Ligase and the ligated

products were transformed into competent *Escherichia coli*, with selection of transformants on agar plates containing selection antibiotic. The selected clones were cultured, the plasmids were recovered and the in-frame sequence of the DNA insert in the plasmids was confirmed by DNA sequencing. The correct clones were subsequently used for expression of the GST-fusion proteins (SEQ ID NO:182); Figure 5B shows the series of full-length P31 (designated P31) (SEQ ID NO:43) and truncated peptides derived from P31 (clones # 101, 102, 103 and 119), (SEQ ID NOS:183, 184, 185, and 186, respectively) full-length PAX2 (designated PAX2) (SEQ ID NO:55) and truncated peptides derived from PAX2 (clones # 104, 105, 106) (SEQ ID NOS:170, 187, and 188, respectively) and full-length DCX8 (DCX8) (SEQ ID NO:23) and series of truncated peptides derived from DCX8 (clones # 107, 108, 109) (SEQ ID NOS:189, 190, and 191, respectively) that were expressed as fusion proteins to GST. The construction of these GST-fusion proteins is shown in Figure 5A. Figure 5C shows the series of full-length P31 (designated P31) (SEQ ID NO:43) and truncated peptides derived from P31 (clones # 103, 110, 119, 111, and 112) (SEQ ID NOS:185, 192, 193, 194, and 195, respectively), full-length PAX2 (designated PAX2) (SEQ ID NO:55) and truncated peptides derived from PAX2 (clones # 106, 113, 114, 115) (SEQ ID NOS:188, 196, 197, and 198, respectively) and full-length SNI10 (designated SNI10) (SEQ ID NO:4) and series of truncated peptides derived from SNI10 (clones # 116, 117, 118) (SEQ ID NOS:199, 200, and 201, respectively) that were expressed as fusion proteins to GST. The construction of these GST-fusion proteins is shown in Figure 5A. (Underlining and bold in Figs. 5A-5C are for orientation of the sequences.)

**Figures 6A-6B.** Figures 6A-6B show the binding of GST and GST-fusion proteins to recombinant hSI and to fixed C2BBel fixed cells as detected by ELISA assays. Figure 6A shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from SNI10 (designated GST-SNI10) and SNI34 (designated GST-SNI34) to

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recombinant hSI. Figure 6B shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from SNI10 (designated GST-SNI10) and SNI34 (designated GST-SNI34) to fixed C2BBel cells.

**5 Figures 7A-7M.** Figures 7A-7M show the binding of GST peptide and truncated fusion proteins to fixed Caco-2 cells, fixed C2BBel cells, and fixed A431 cells or to recombinant GIT transport receptors D2H, HPT1, hPEPT1 or to BSA using increasing concentrations (expressed as  $\mu\text{g/ml}$  on the X-axis) of the control GST protein and the GST-fusion proteins, as detected by ELISA assays. Figure 7A shows the binding of the control protein GST, which does not contain a fusion peptide, and the series of GST-fusion proteins from P31 including the fusion to full-length P31 peptide (designated P31) (SEQ ID NO:43) and clone # 101 (designated P31,101), clone # 102 (designated P31, 102) and clone # 103 (designated P31,103). Figure 7B shows the binding of the control protein GST, which does not contain a fusion peptide, and the series of GST-fusion proteins from PAX2 including the fusion to full-length PAX2 peptide (designated PAX2) and clone # 104 (designated PAX2,104), clone # 105 (designated PAX2, 105) and clone # 106 (designated PAX2,106) (SEQ ID NOS:55, 170, 187, and 188, respectively). Figure 7C shows the binding of the control protein GST, which does not contain a fusion peptide, and the series of GST-fusion proteins from DCX8 including the fusion to full-length DCX8 peptide (designated DCX8) and clone # 107 (designated DCX8,107), clone # 108 (designated DCX8, 108) and clone # 109 (designated DCX8,109) (SEQ ID NOS:23, 189, 190, and 191, respectively). Figure 7D shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from DCX8 (designated GST-DCX8) and DCX11 (designated GST-DCX11) to recombinant D2H. Figure 7E shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from DCX8 (designated GST-DCX8) and DCX11 (designated GST-DCX11) to fixed C2BBel cells. Figure 7F shows the binding of the control protein GST, which does not contain a fusion

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peptide, and the GST-fusion proteins from P31 (designated GST-P31) and 5PAX5 (designated GST-5PAX5) to recombinant hPEPT1. Figure 7G shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from P31 (designated GST-P31) and 5PAX5 (designated GST-5PAX5) to fixed C2BBel cells. Figure 7H shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from HAX42 (designated GST-HAX42) and PAX2 (designated GST-PAX2) to recombinant HPT1. Figure 7I shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from HAX42 (designated GST-HAX42) and PAX2 (designated GST-PAX2) to fixed C2BBel cells. Figure 7J shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from P31 (designated GST-P31) and truncated derivatives clone # 101 (designated GST-P31-101), clone # 102 (designated GST-P31-102), clone # 103 (designated GST-P31-103) to either recombinant hPEPT1 or to BSA. Figure 7K shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from P31 (designated GST-P31) and truncated derivatives clone # 101 (designated GST-P31-101), clone # 102 (designated GST-P31-102), clone # 103 (designated GST-P31-103) to either fixed C2BBel cells or to fixed A431 cells. Figure 7L shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from PAX2 (designated GST-PAX2) and truncated derivatives clone # 104 (designated GST-PAX2-104), clone # 105 (designated GST-PAX2-105), clone # 106 (designated GST-PAX2-106) to either recombinant hPEPT1 or to BSA. Figure 7M shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from PAX2 (designated GST-PAX2) and truncated derivatives clone # 106 (designated GST-PAX2-106) to either fixed Caco-2 cells or to fixed A431 cells.

**Figures 8A-8D.** Figure 8 shows the transport of GST or GST-peptide fusion derivatives across polarized Caco-2 cells in

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an apical to basolateral direction as a function of time (1-4 hours) as detected by ELISA assays. Figure 8A shows the transport of either GST, the GST fusion to full-length P31 peptide (designated P31) (SEQ ID NO:43) and the GST clone derivative clone # 103 (designated P31.103) across polarized  
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Caco-2 cells in an apical to basolateral as a function of time (in hours) following initial administration of the proteins to the apical medium of polarized Caco-2 cells. The line designated No Protein corresponds to control assays in  
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which buffer control was applied to the apical medium of polarized Caco-2 cells followed by sampling of the basolateral medium as a function of time (hours) and assay for GST by the ELISA assay. Figure 8B shows the transport of either GST, the GST fusion to full-length PAX2 peptide  
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(designated PAX2) and the GST clone derivative clone # 106 (designated PAX2.106) across polarized Caco-2 cells in an apical to basolateral as a function of time (in hours) following initial administration of the proteins to the apical medium of polarized Caco-2 cells. The line designated  
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No Protein corresponds to control assays in which buffer control was applied to the apical medium of polarized Caco-2 cells followed by sampling of the basolateral medium as a function of time (hours) and assay for GST by the ELISA assay. Figure 8C shows the transport of either GST, the GST  
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fusion to full-length DCX8 peptide (designated DCX8), and the GST clone derivatives clone # 107 (designated DCX8.107) and clone # 109 (designated DCX8.109) across polarized Caco-2 cells in an apical to basolateral as a function of time (in hours) following initial administration of the proteins to  
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the apical medium of polarized Caco-2 cells. The line designated No Protein corresponds to control assays in which buffer control was applied to the apical medium of polarized Caco-2 cells followed by sampling of the basolateral medium as a function of time (hours) and assay for GST by the ELISA  
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assay. Figure 8D shows the amount of the GST and GST-fusion proteins (GST fusions to P31, P31-103, PAX2, PAX2.106, DCX8, DCX8-107, DCX8-109), used in the experiments shown in panels

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A-C above, in the apical medium of the polarized Caco-2 cells as detected by ELISA assay.

**Figures 9A-9B.** Figures 9A-9B show the inhibition of GST-P31 binding to C2BBel fixed cells with varying concentration of 5 competitors while holding the concentration of GST-P31 constant at 0.015  $\mu$ M; the peptide competitors are ZElan024 which is the dansylated peptide version of P31 (SEQ ID NO:43) and ZElan044, ZElan049 and ZElan050 which are truncated, dansylated pieces of P31 (SEQ ID NO:43). Data is presented 10 as O.D. versus peptide concentration (Figure 9A) and as percent inhibition of GST-P31 binding versus peptide concentration (Figure 9B).

**Figures 10A-10C.** Figures 10A-10C present a compilation of the results of competition ELISA studies of GST-P31, GST-15 PAX2, GST-SNi10 and GST-HAX42 versus listed dansylated peptides on fixed C2BBel cells ("Z" denotes  $\epsilon$ -amino dansyl lysine). The pI of the dansylated peptides is also included. Estimated IC<sub>50</sub> values are in  $\mu$ M and where present, IC<sub>50</sub> ranges refer to results from multiple assays. If the IC<sub>50</sub> value 20 could not be determined, a ">" or "<" symbol is used. The GST/C2BBel column shows GST protein binding to fixed C2BBel cells.

**Figures 11A-11B.** Figure 11A shows the transport of GST or GST-peptide fusion derivatives across polarized Caco-2 cells 25 in an apical to basolateral direction at 0, 0.5, 2 and 4 hours as detected by ELISA assays and described elsewhere in the text in full detail. The proteins used in the assay included GST, GST-P31 fusion, GST-5PAX5 fusion, GST-DCX8 fusion, GST-DCX11 fusion, GST-PAX2 fusion, GST-HAX42 fusion, 30 GST-SNi34 fusion and GST-SNi10 fusion. The column designated No protein refers to control experiments in which buffer was applied to the apical medium of the cells and ELISA assay was performed on the corresponding basolateral medium of these cells at 0, 0.5, 2 and 4 hours post buffer addition. Figure 35 11B shows the internalization of GST or GST-peptide fusion derivatives within polarized Caco-2 cells following administration of the GST or GST-fusion protein derivatives

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to the apical medium of polarized Caco-2 cells and subsequent recovery of the cells from the transwells and detection of the GST or GST fusions within the recovered cell lysates as detected by ELISA assays and as described elsewhere in the text in full detail. The proteins used in the assay included GST, GST-P31 fusion, GST-5PAX5 fusion, GST-DCX8 fusion, GST-DCX11 fusion, GST-PAX2 fusion, GST-HAX42 fusion, GST-SNi34 fusion and GST-SNi10 fusion. The column designated No protein refers to control experiments in which buffer was applied to the apical medium of the cells and ELISA assay was performed on the corresponding cell lysates of these cells at the end of the experiment.

**Figure 12.** Figure 12 shows the binding of GST and GST-fusion proteins to fixed Caco-2 cells, and the corresponding proteins following digestion with the protease Thrombin which cleaves at a recognition site between the GST portion and the fused peptide portion of the GST-fusion protein. The symbol "-" refers to proteins which were not digested with thrombin and the symbol "+" refers to proteins which were digested with thrombin prior to use in the binding assay. The binding of the proteins to the fixed Caco-2 cells was detected by ELISA assays.

**Figures 13A-13B.** Figures 13A-13B show binding of peptide-coated nanoparticles to fixed Caco-2 cells.

**Figures 14A-14B.** Figures 14A-14B show the binding of (A) dansylated peptide SNi10 to the purified hSI receptor and BSA and (B) dansylated peptides and peptide-loaded insulin-containing PLGA particles to fixed C2BBel cells. Figure 14B depicts binding of dansylated peptides corresponding to P31 (SEQ ID NO:43), PAX2, HAX42, and SNi10 to fixed C2BBel cells, as well as the insulin-containing PLGA particles adsorbed with each of these peptides. Data is presented with background subtracted.

**Figures 15A-15B.** Figure 15 shows the binding of peptide-coated particles to A) S100 and B) P100 fractions harvested from Caco-2 cells. The dilution series 1:2 - 1:64 represents particle concentrations in the range 0.0325-0.5  $\mu\text{g}/\text{well}$ .

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Data is presented with background subtracted. The particles are identified as follows: 939, no peptide; 1635, scrambled PAX2; 1726, P31 D-Arg 16-mer (ZElan053); 1756, HAX42; 1757, PAX2; 1758, HAX42/PAX2.

5 **Figures 16A-16B.** Figure 16 shows the binding of dansylated peptides to P100 fractions harvested from Caco-2 cells. Peptides were assayed in the range 0.0032-2.5 µg/well. Data is presented with background subtracted. A) HAX42, P31 D-form (ZElan 053) and scrambled PAX2; B) PAX2, HAX42 and  
10 scrambled PAX2.

**Figures 17A-17B.** Figures 17A and 17B show (A) the systemic blood glucose and (B) insulin levels following intestinal administration of control (PBS); insulin solution; insulin particles; all 8 peptide mix particles and study group  
15 peptide-particles according to this invention (100iu insulin loading).

**Figures 18A-18B.** Figures 18A and 18B show the (A) systemic blood glucose and (B) insulin levels following intestinal administration of control (PBS); insulin solution; insulin  
20 particles and study group peptide-particles according to this invention (300iu insulin loading).

**Figure 19.** Figure 19 shows the enhanced plasma levels of leuprolide upon administration of P31 (SEQ ID NO:43) and PAX2 coated nanoparticles loaded with leuprolide relative to  
25 subcutaneous injection. Group 1 was administered leuprolide acetate (12.5 µg) subcutaneously. Group 2 was administered intraduodenally uncoated leuprolide acetate particles (600 µg, 1.5 ml). Group 3 was intraduodenally administered leuprolide acetate particles coated with PAX2 (600 µg; 1.5  
30 ml). Group 4 was administered intraduodenally leuprolide acetate particles coated with P31 (SEQ ID NO:43) (600 µg, 1.5 ml).

**Figure 20.** Figure 20 lists P31 (SEQ ID NO:43) known protein homologies.

35 **Figures 21A-21C.** Figures 21A-21C list DCX8 known protein homologies.

**Figure 22.** Figure 22 lists DAB10 known protein homologies.

19

**Figure 23.** Figure 23 shows the DNA sequence (SEQ ID NO:211) and the corresponding amino acid sequence (SEQ ID NO:212) for glutathione S-transferase (Smith and Johnson, 1988, Gene 7:31-40).

5

**5. DETAILED DESCRIPTION OF THE INVENTION**

The present invention relates to proteins (e.g., peptides) that bind to GIT transport receptors and nucleic acids that encode such proteins. The invention further  
10 relates to fragments and other derivatives of such proteins. Nucleic acids encoding such fragments or derivatives are also within the scope of the invention. The invention further relates to fragments (and derivatives and analogs thereof) of GIT transport receptor-binding peptides which comprise one or  
15 more domains of the GIT transport receptor-binding peptides.

The invention also relates to derivatives of GIT transport receptor-binding proteins and analogs of the invention which are functionally active, i.e., they are capable of displaying one or more known functional activities  
20 associated with a full-length GIT transport receptor-binding peptide. Such functional activities include but are not limited to ability to bind to a GIT transport receptor, antigenicity [ability to bind (or compete with peptides for binding) to an anti-GIT transport receptor-binding peptide  
25 antibody], immunogenicity (ability to generate antibody which binds to GIT transport receptor-binding peptide), etc.

Production of the foregoing proteins and derivatives, by, e.g., recombinant methods, is also provided.

Antibodies to GIT transport receptor-binding  
30 proteins, derivatives and analogs, are additionally provided.

The present invention also relates to therapeutic and diagnostic methods and compositions based on GIT transport receptor-binding proteins and nucleic acids.

The invention is illustrated by way of examples  
35 *infra*.

20

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

5           **5.1. GIT Transport Receptor-Binding Peptides,  
              Derivatives and Analogs**

              The invention relates to peptides that bind GIT  
transport receptors and derivatives (including but not  
limited to fragments) and analogs thereof. In specific  
10       embodiments, of the present invention, such peptides that  
bind to GIT transport receptor include but are not limited to  
those containing as primary amino acid sequences, all or part  
of the amino acid sequences substantially as depicted in  
Table 7 (SEQ ID NOS:1-55). Nucleic acids encoding such  
15       peptides, derivatives and peptide analogs are also provided.  
In one embodiment, the GIT transport receptor-binding  
peptides are encoded by the nucleic acids having the  
nucleotide sequences set forth in Table 8 *infra* (SEQ ID  
NOS:56-109). Proteins whose amino acid sequence comprise, or  
20       alternatively, consist of SEQ ID NOS:1-55 or a portion  
thereof that mediates binding to a GIT transport receptor are  
provided.

              The production and use of derivatives and analogs  
related to GIT transport receptor-binding peptides are within  
25       the scope of the present invention. In a specific  
embodiment, the derivative or analog is functionally active,  
*i.e.*, capable of exhibiting one or more functional activities  
associated with a full-length GIT transport receptor-binding  
peptide. For example, such derivatives or analogs which have  
30       the desired immunogenicity or antigenicity can be used, in  
immunoassays, for immunization, etc. A specific embodiment  
relates to a GIT transport receptor-binding peptide fragment  
that can be bound by an anti-GIT transport receptor-binding  
peptide antibody. In a preferred aspect, the derivatives or  
35       analogs have the ability to bind to a GIT transport receptor.  
Derivatives or analogs of GIT transport receptor-binding  
peptides can be tested for the desired activity by procedures

known in the art, including binding to a GIT transport receptor domain or to Caco-2 cells, *in vitro*, or to intestinal tissue, *in vivo*. (See the Examples *infra*.)

In particular, derivatives can be made by altering

5 GIT transport receptor-binding peptide sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other nucleotide sequences which encode substantially the same amino acid sequence may be used

10 in the practice of the present invention. These include but are not limited to nucleotide sequences which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the GIT

15 transport receptor-binding peptide derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a GIT transport receptor-binding peptide including altered sequences in which functionally equivalent

20 amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent

25 alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and

30 methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and

35 glutamic acid.

In a specific embodiment of the invention, proteins consisting of or, alternatively, comprising all or a fragment

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of a GIT transport receptor-binding peptide consisting of at least 5, 10, 15, 20, 25, 30 or 35 (contiguous) amino acids of the full-length GIT transport receptor-binding peptide are provided. In a specific embodiment, such proteins are not more than 20, 30, 40, 50, or 75 amino acids in length. Derivatives or analogs of GIT transport receptor-binding peptides include but are not limited to those molecules comprising regions that are substantially homologous to GIT transport receptor-binding peptides or fragments thereof (e.g., at least 50%, 60%, 70%, 80% or 90% identity) (e.g., over an identical size sequence or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art) or whose encoding nucleic acid is capable of hybridizing to a coding GIT transport receptor-binding peptide sequence, under stringent, moderately stringent, or nonstringent conditions.

In a specific embodiment, the GIT transport receptor-binding derivatives of the invention are not known proteins with homology to the GIT transport receptor-binding peptides of the invention or portions thereof.

The GIT transport receptor-binding peptide derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned GIT transport receptor-binding peptide gene sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog of GIT transport receptor-binding peptides, care should be taken to ensure that the modified gene remains within the same translational reading frame uninterrupted by translational

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stop signals, in the gene region where the desired GIT transport receptor-binding peptides activity is encoded.

Additionally, nucleic acid sequences encoding the GIT transport receptor-binding peptides can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), use of TAB® linkers (Pharmacia), use of PCR primers containing mutation(s) for use in amplification, etc.

Manipulations of GIT transport receptor-binding peptide sequences may also be made at the protein level. Included within the scope of the invention are GIT transport receptor-binding peptide fragments or other derivatives or analogs which are differentially modified during or after translation or chemical synthesis, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH<sub>4</sub>; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc. In a specific embodiment, the amino- and/or carboxy-termini are modified.

In addition, GIT transport receptor-binding peptides and analogs and derivatives thereof can be chemically synthesized. For example, a peptide corresponding to all or a portion of a GIT transport receptor-binding peptide which comprises the desired domain or which mediates the desired activity *in vitro*, can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical

amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the GIT transport receptor-binding peptide sequence. Non-classical amino acids include but are not limited to the D-isomers of the common

5 amino acids,  $\alpha$ -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid,  $\gamma$ -Abu,  $\epsilon$ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine,

10 phenylglycine, cyclohexylalanine,  $\beta$ -alanine, fluoro-amino acids, designer amino acids such as  $\beta$ -methyl amino acids, C $\alpha$ -methyl amino acids, N $\alpha$ -methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

15 In a specific embodiment, the GIT transport receptor-binding peptide derivative is a chimeric, or fusion, peptide comprising a GIT transport receptor-binding peptide or fragment thereof (preferably consisting of at least a domain or motif of the GIT transport receptor-binding

20 peptide, or at least 6, 10, 15, 20, 25, 30 or all amino acids of the GIT transport receptor-binding peptides or a binding portion thereof) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different peptide. In one embodiment, such a chimeric peptide is

25 produced by recombinant expression of a nucleic acid encoding the protein (comprising a transport receptor-coding sequence joined in-frame to a coding sequence for a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired

30 amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, e.g., by use of a peptide synthesizer. Chimeric

35 genes comprising portions of GIT transport receptor fused to any heterologous protein-encoding sequences may be constructed. A specific embodiment relates to a chimeric

25



protein comprising a fragment of GIT transport receptor-binding peptides of at least six amino acids.

In another specific embodiment, the GIT transport receptor-binding peptide derivative is a molecule comprising  
5 a region of homology with a GIT transport receptor-binding peptide. By way of example, in various embodiments, a first protein region can be considered "homologous" to a second protein region when the amino acid sequence of the first region is at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, or  
10 95% identical, when compared to any sequence in the second region of an equal number of amino acids as the number contained in the first region or when compared to an aligned sequence of the second region that has been aligned by a computer homology program known in the art. For example, a  
15 molecule can comprise one or more regions homologous to a GIT transport receptor-binding peptide domain (see *infra*) or a portion thereof.

The GIT transport receptor-binding proteins and derivatives thereof of the invention can be assayed for  
20 binding activity by suitable *in vivo* or *in vitro* assays, e.g., as described in the examples *infra* and/or as will be known to the skilled artisan.

Other specific embodiments of derivatives and analogs are described in the subsection below and examples  
25 sections *infra*.

#### **5.2. Motifs/Derivatives of GIT Transport Receptor-Binding Peptides Containing One or More Domains of The Protein**

In a specific embodiment, the invention relates to  
30 GIT transport receptor-binding peptide derivatives and analogs, in particular GIT transport receptor-binding peptide fragments and derivatives of such fragments, that comprise, or alternatively consist of, one or more domains of a GIT transport receptor-binding peptide. In particular, examples  
35 of such domains are identified in the examples *infra*.

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### 5.3. Synthesis of Peptides

The peptides and derivatives of the present invention may be chemically synthesized or synthesized using recombinant DNA techniques.

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#### 5.3.1. Procedure For Solid Phase Synthesis

Peptides may be prepared chemically by methods that are known in the art. For example, in brief, solid phase peptide synthesis consists of coupling the carboxyl group of  
10 the C-terminal amino acid to a resin and successively adding N-alpha protected amino acids. The protecting groups may be any known in the art. Before each new amino acid is added to the growing chain, the protecting group of the previous amino acid added to the chain is removed. The coupling of amino  
15 acids to appropriate resins is described by Rivier et al., U.S. Patent No. 4,244,946. Such solid phase syntheses have been described, for example, by Merrifield, 1964, J. Am. Chem. Soc. 85:2149; Vale et al., 1981, Science 213:1394-1397; Marki et al., 1981, J. Am. Chem. Soc. 103:3178 and in U.S.  
20 Patent Nos. 4,305,872 and 4,316,891. In a preferred aspect, an automated peptide synthesizer is employed.

By way of example but not limitation, peptides can be synthesized on an Applied Biosystems Inc. ("ABI") model 431A automated peptide synthesizer using the "Fastmoc"  
25 synthesis protocol supplied by ABI, which uses 2-(1H-Benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate ("HBTU") (R. Knorr et al., 1989, Tet. Lett., 30:1927) as coupling agent. Syntheses can be carried out on 0.25 mmol of commercially available  
30 4-(2',4'-dimethoxyphenyl)-(9-fluorenyl-methoxycarbonyl)-aminomethyl)-phenoxy polystyrene resin ("Rink resin" from Advanced ChemTech) (H. Rink, 1987, Tet. Lett. 28:3787). Fmoc amino acids (1 mmol) are coupled according to the Fastmoc protocol. The following side chain  
35 protected Fmoc amino acid derivatives are used:  
FmocArg(Pmc)OH; FmocAsn(Mbh)OH; FmocAsp(<sup>t</sup>Bu)OH;  
FmocCys(Acm)OH; FmocGlu(<sup>t</sup>Bu)OH; FmocGln(Mbh)OH; FmocHis(Tr)OH;

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FmocLys(Boc)OH; FmocSer(<sup>t</sup>Bu)OH; FmocThr(<sup>t</sup>Bu)OH;  
FmocTyr(<sup>t</sup>Bu)OH. [Abbreviations: Ac<sub>m</sub>, acetamidomethyl; Boc,  
tert-butoxycarbonyl; <sup>t</sup>Bu, tert-butyl; Fmoc,  
9-fluorenylmethoxycarbonyl; Mb<sub>h</sub>, 4,4'-dimethoxybenzhydryl;  
5 Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Tr, trityl].

Synthesis is carried out using N-methylpyrrolidone  
(NMP) as solvent, with HBTU dissolved in  
N,N-dimethylformamide (DMF). Deprotection of the Fmoc group  
is effected using approximately 20% piperidine in NMP. At  
10 the end of each synthesis the amount of peptide present is  
assayed by ultraviolet spectroscopy. A sample of dry peptide  
resin (about 3-10 mg) is weighed, then 20% piperidine in DMA  
(10 ml) is added. After 30 min sonication, the UV  
(ultraviolet) absorbance of the dibenzofulvene-piperidine  
15 adduct (formed by cleavage of the N-terminal Fmoc group) is  
recorded at 301 nm. Peptide substitution (in mmol g<sup>-1</sup>) can be  
calculated according to the equation:

substitution = 
$$\frac{A \times v}{7800 \times w} \times 1000$$

20 where A is the absorbance at 301 nm, v is the volume of 20%  
piperidine in DMA (in ml), 7800 is the extinction coefficient  
(in mol<sup>-1</sup>dm<sup>3</sup>cm<sup>-1</sup>) of the dibenzofulvene-piperidine adduct, and  
w is the weight of the peptide-resin sample (in mg).

25 Finally, the N-terminal Fmoc group is cleaved using  
20% piperidine in DMA, then acetylated using acetic anhydride  
and pyridine in DMA. The peptide resin is thoroughly washed  
with DMA, CH<sub>2</sub>Cl<sub>2</sub> and finally diethyl ether.

30 **5.3.2. Cleavage And Deprotection**

By way of example but not limitation, cleavage and  
deprotection can be carried out as follows: The air-dried  
peptide resin is treated with ethylmethyl-sulfide (EtSMe),  
ethanedithiol (EDT), and thioanisole (PhSMe) for  
35 approximately 20 min. prior to addition of 95% aqueous  
trifluoroacetic acid (TFA). A total volume of approximately  
50 ml of these reagents per gram of peptide-resin is used.

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The following ratio is used: TFA:EtSMe:EDT:PhSMe (10:0.5:0.5:0.5). The mixture is stirred for 3 h at room temperature under an atmosphere of N<sub>2</sub>. The mixture is filtered and the resin washed with TFA (2 x 3 ml). The combined filtrate is evaporated *in vacuo*, and anhydrous diethyl ether added to the yellow/orange residue. The resulting white precipitate is isolated by filtration. See King et al., 1990, Int. J. Peptide Protein Res. 36:255-266 regarding various cleavage methods.

10

### 5.3.3. Purification of the Peptides

Purification of the synthesized peptides can be carried out by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography, high performance liquid chromatography (HPLC)), centrifugation, differential solubility, or by any other standard technique.

15

### 5.3.4. Biological Peptide Libraries

Biological peptide libraries can be used to express and identify peptides that bind to GIT transport receptors. According to this second approach, involving recombinant DNA techniques, peptides can, by way of example, be expressed in biological systems as either soluble fusion proteins or viral capsid proteins.

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#### 5.3.4.1. Methods To Identify Binders: Construction Of Libraries

In a specific embodiment, the peptides of the invention that specifically bind to GIT transport receptors are identified by screening a random peptide library by contacting the library with a ligand selected from among HPT1, hPEPT1, D2H, or hSI (or a molecule consisting essentially of an extracellular domain thereof or fragment of the domain) to identify members of the library that specifically bind to the ligand.

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**5.3.4.3. Methods to Identify Binders:  
Screening Libraries**

5 Once a suitable random peptide library has been constructed (or otherwise obtained), the library is screened to identify peptides having binding affinity for the GIT transport receptor, e.g., HPT1, hPEPT1, D2H, or hSI. In a preferred aspect, the library is a TSAR library (see U.S. Patent No. 5,498,538 dated March 12, 1996 and PCT Publication WO 94/18318 dated August 18, 1994, both of which are incorporated by reference herein in their entireties).

10 Screening the libraries can be accomplished by any of a variety of methods known to those of skill in the art. See, e.g., the following references, which disclose screening of peptide libraries: Parmley and Smith, 1989, Adv. Exp. Med. Biol. 251: 215-218; Scott and Smith, 1990, Science 249: 386-390; Fowlkes et al., 1992, BioTechniques 13: 422-427; Oldenburg et al., 1992, Proc. Natl. Acad. Sci. USA 89: 5393-5397; Yu et al., 1994, Cell 76: 933-945; Staudt et al., 1988, Science 241: 577-580; Bock et al., 1992, Nature 355: 564-566; Tuerk et al., 1992, Proc. Natl. Acad. Sci. USA 89: 6988-6992; Ellington et al., 1992, Nature 355: 850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al.; and Rebar and Pabo, 1993, Science 263: 671-673. See also PCT publication WO 94/18318, dated August 18, 1994.

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One of ordinary skill in the art will recognize that, with suitable modifications, the screening methods described below would be suitable for a wide variety of biological expression libraries.

30 Once a library has been constructed or otherwise obtained, the library is screened to identify binding molecules having specific binding affinity for a ligand for a GIT transport receptor preferably selected from among HPT1, hPEPT1, D2H, or hSI.

35 Screening the libraries can be accomplished by any of a variety of methods known to those of skill in the art. Exemplary screening methods are described in Fowlkes et al.,

1992, BioTechniques, 13:422-427 and include contacting the vectors with an immobilized target ligand and harvesting those vectors that bind to said ligand. Such useful screening methods, are designated "panning" methods. In  
5 panning methods useful to screen the present libraries, the target ligand can be immobilized on plates, beads (such as magnetic beads), sepharose, beads used in columns, etc. If desired, the immobilized target ligand can be "tagged", e.g., using labels such as biotin, fluoroscein isothiocyanate,  
10 rhodamine, etc. e.g. for FACS sorting. Panning is also disclosed in Parmley, S.F. and Smith, G.P., 1988, Gene 73: 305-318.

In a particular embodiment of the invention, the library can be screened with a recombinant receptor domain.  
15 In another embodiment, the library can be screened successively with receptor domains and then on CaCO-2 cells.

For screening of the peptide libraries *in vitro*, the solvent requirements involved in screening are not limited to aqueous solvents; thus, nonphysiological binding  
20 interactions and conditions different from those found *in vivo* can be exploited.

Screening a library can be achieved using a method comprising a first "enrichment" step and a second filter lift as follows. The following description is given by way of  
25 example, not limitation.

Binders from an expressed library (e.g., in phage) capable of binding to a given ligand ("positives") are initially enriched by one or two cycles of panning or affinity chromatography. A microtiter well is passively  
30 coated with the ligand (e.g., about 10  $\mu$ g in 100  $\mu$ l). The well is then blocked with a solution of BSA to prevent non-specific adherence of the phage of the library to the plastic surface. For example, about  $10^{11}$  phage particles expressing peptides are then added to the well and incubated for several  
35 hours. Unbound phage are removed by repeated washing of the plate, and specifically bound phage are eluted using an acidic glycine-HCl solution or other elution buffer. The

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eluted phage solution is neutralized with alkali, and amplified, e.g., by infection of *E. coli* and plating on large petri dishes containing Luria broth (LB) in agar. Amplified cultures expressing the binding peptides are then titered and the process repeated. Alternatively, the ligand can be covalently coupled to agarose or acrylamide beads using commercially available activated bead reagents. The phage solution is then simply passed over a small column containing the coupled bead matrix which is then washed extensively and eluted with acid or other eluant. In either case, the goal is to enrich the positives to a frequency of about  $> 1/10^5$ .

Following enrichment, a filter lift assay is conducted. For example, when specific binders are expressed in phage, approximately  $1-2 \times 10^5$  phage are added to 500  $\mu$ l of log phase *E. coli* and plated on a large Luria Broth-agarose plate with 0.7% agarose in broth. The agarose is allowed to solidify, and a nitrocellulose filter (e.g., 0.45  $\mu$ ) is placed on the agarose surface. A series of registration marks is made with a sterile needle to allow re-alignment of the filter and plate following development as described below. Phage plaques are allowed to develop by overnight incubation at 37 °C (the presence of the filter does not inhibit this process). The filter is then removed from the plate with phage from each individual plaque adhered *in situ*. The filter is then exposed to a solution of BSA or other blocking agent for 1-2 hours to prevent non-specific binding of the ligand (or "probe").

The probe itself is labeled, for example, either by biotinylation (using commercial NHS-biotin) or direct enzyme labeling, e.g., with horse radish peroxidase or alkaline phosphatase. Probes labeled in this manner are indefinitely stable and can be re-used several times. The blocked filter is exposed to a solution of probe for several hours to allow the probe to bind *in situ* to any phage on the filter displaying a peptide with significant affinity to the probe. The filter is then washed to remove unbound probe, and then developed by exposure to enzyme substrate solution (in the

33



case of directly labeled probe) or further exposed to a solution of enzyme-labeled avidin (in the case of biotinylated probe). Positive phage plaques are identified by localized deposition of colored enzymatic cleavage product  
5 on the filter which corresponds to plaques on the original plate. The developed filter is simply realigned with the plate using the registration marks, and the "positive" plaques are cored from the agarose to recover the phage. Because of the high density of plaques on the original plate,  
10 it may be difficult to isolate a single plaque from the plate on the first pass. Accordingly, phage recovered from the initial core can be re-plated at low density and the process can be repeated to allow isolation of individual plaques and hence single clones of phage.

15               Successful screening experiments are optimally conducted using 3 rounds of serial screening. The recovered cells are then plated at a low density to yield isolated colonies for individual analysis. The individual colonies are selected and used to inoculate LB culture medium  
20 containing ampicillin. After overnight culture at 37°C, the cultures are then spun down by centrifugation. Individual cell aliquots are then retested for binding to the target ligand attached to the beads. Binding to other beads having attached thereto a non-relevant ligand, can be used as a  
25 negative control.

One aspect of screening the libraries is that of elution. The following discussion is applicable to any system where the random peptide is expressed on a surface fusion molecule. It is conceivable that the conditions that  
30 disrupt the peptide-target interactions during recovery of the phage are specific for every given peptide sequence from a plurality of proteins expressed on phage. For example, certain interactions may be disrupted by acid pH but not by basic pH, and vice versa. Thus, it may be desirable to test  
35 a variety of elution conditions (including but not limited to pH 2-3, pH 12-13, excess target in competition, detergents, mild protein denaturants, urea, varying temperature, light,

presence or absence of metal ions, chelators, etc.) and compare the primary structures of the binding proteins expressed on the phage recovered for each set of conditions to determine the appropriate elution conditions for each  
5 ligand/binding protein combination. Some of these elution conditions may be incompatible with phage infection because they are bactericidal and will need to be removed by dialysis (i.e., dialysis bag, Centricon/Amicon microconcentrators).

In a preferred embodiment, a phage display library  
10 of random peptides is screened to select phage expressing peptides that bind to a GIT transport receptor. Preferably, a first step is to isolate a preselected phage library. The "preselected phage library" is a library consisting of a subpopulation of a phage display library. This subpopulation  
15 can be formed by initially screening against either a target GIT transport receptor (or domain thereof) so as to permit the selection of a subpopulation of phages which specifically bind to the receptor. Alternatively, the subpopulation can be formed by screening against a target cell or cell type or  
20 tissue type or tissue barrier of the gastro-intestinal tract, so as to permit the selection of a subpopulation of phages which either bind specifically to the target cell or target cell type or target tissue or target tissue barrier, or which binds to and/or is transported across (or between) the target  
25 cell or target cell type or target tissue or target tissue barrier either *in situ* or *in vivo*. This preselected phage library or subpopulation of selected phages can also be rescreened against the target GIT transport receptor, permitting the further selection of a subpopulation of phages  
30 which bind to the GIT transport receptor or target cell or target cell type or target tissue or target tissue barrier or which bind to and/or is transported across the target cell, target tissue or target tissue barrier either *in situ* or *in vivo*. Such rescreening can be repeated from zero to 30 times  
35 with each successive "pre-selected phage library" generating additional pre-selected phage libraries.

35

5 In a preferred embodiment, a preselected phage library binding a ligand that is a GIT transport receptor preferably selected from among HPT1, hPEPT1, D2H, or hSI is obtained by an *in vitro* screening step as described above, and then the phage are optionally further characterized using *in vitro* assays consisting of binding phage directly to the receptor domain of interest or, alternatively, to Caco-2 cells or using *in vivo* assays. In another preferred embodiment, *in vivo* assays are used that measure uptake of phage by intestinal tissue or, alternatively, through the GIT. In alternative embodiments, such further *in vitro* or *in vivo* assays can be used as the initial screening step.

*In vivo* assays that may be used are described in the examples *infra*.

15

#### **5.4. Generation of Antibodies to GIT Transport Receptor-Binding Peptides and Derivatives Thereof**

20 According to the invention, a GIT transport receptor-binding peptide, fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library.

25 Various procedures known in the art may be used for the production of polyclonal antibodies to a GIT transport receptor-binding peptide or derivative or analog. For the production of antibody, various host animals can be immunized by injection with the native GIT transport receptor-binding peptides, or a synthetic version, or derivative (e.g.,  
30 fragment) thereof, including but not limited to rabbits, mice, rats, fowl, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface  
35 active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet

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hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed  
5 toward a GIT transport receptor-binding peptide or analog thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497),  
10 as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an  
15 additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci.  
20 U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). According to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984,  
25 Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for GIT transport receptor-binding peptides together with genes from a human antibody molecule of  
30 appropriate biological activity can be used.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce GIT transport receptor-binding peptide-specific single chain antibodies. An  
35 additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow

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rapid and easy identification of monoclonal Fab fragments with the desired specificity for GIT transport receptor-binding peptides, derivatives, or analogs.

Antibody fragments which contain the idiotype of  
5 the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the  $F(ab')_2$  fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the  $F(ab')_2$   
10 fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in  
15 the art, e.g. ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific domain of a GIT transport receptor-binding peptide, one may assay generated hybridomas for a product which binds to a GIT transport receptor-binding peptide fragment containing such a  
20 domain.

Antibodies specific to a domain of a GIT transport receptor-binding peptide are also provided.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of  
25 the GIT transport receptor-binding peptide sequences of the invention, e.g., for imaging these peptides after *in vivo* administration (e.g., to monitor treatment efficacy), measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc. For instance,  
30 antibodies or antibody fragments specific to a domain of a GIT transport receptor-binding peptide or to a derivative of a peptide, such as a dansyl group or some other epitope introduced into the peptide, can be used to 1) identify the presence of the peptide on a nanoparticle or other substrate;  
35 2) quantify the amount of peptide on the nanoparticle;  
3) measure the level of the peptide in appropriate physiological samples; 4) perform immunohistology on tissue

samples; 5) image the peptide after *in vivo* administration; 6) purify the peptide from a mixture using an immunoaffinity column or 7) bind or fix the peptide to the surface of nanoparticle. This last use envisions attaching the antibody 5 (or fragment of the antibody) to the surface of drug-loaded nanoparticles or other substrate and then incubating this conjugate with the peptide. This procedure results in binding of the peptide in a certain fixed orientation, resulting in a particle that contains the peptide bound to 10 the antibody in such a way that the peptide is fully active.

Abtides (or **Antigen binding peptides**) specific to a domain of a GIT transport receptor-binding peptide or to a derivative of a peptide, such as a dansyl group or some other epitope introduced into the peptide, can be used for the same  
15 seven purposes identified above for antibodies.

### 5.5. Assays of GIT Transport Receptor-Binding Peptides, Derivatives and Analogs

The functional activity of GIT transport receptor-binding peptides, derivatives and analogs can be assayed by various methods.

In a preferred embodiment, in which binding to a GIT transport receptor is being assayed, the binding can be assayed by *in vivo* or *in vitro* assays such as described in the examples *infra*, or by other means that are known in the art.

In another embodiment, where one is assaying for the ability to bind or compete with full-length GIT transport receptor-binding peptide for binding to anti-GIT transport receptor-binding peptide antibody, various immunoassays known in the art can be used, including but not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western

3d.

blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labelled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

Other methods will be known to the skilled artisan and are within the scope of the invention.

#### 5.6. Uses

The invention provides compositions comprising the GIT transport receptor-binding proteins of the invention bound to a material comprising an active agent. Such compositions have use in targeting the active agent to the GIT and/or in facilitating transfer through the lumen of the GIT into the systemic circulation. Where the active agent is an imaging agent, such compositions can be administered in vivo to image the GIT (or particular transport receptors thereof). Other active agents include but are not limited to: any drug or antigen or any drug- or antigen-loaded or drug- or antigen-encapsulated nanoparticle, microparticle, liposome, or micellar formulation capable of eliciting a biological response in a human or animal. Examples of drug- or antigen-loaded or drug- or antigen-encapsulated formulations include those in which the active agent is encapsulated or loaded into nano- or microparticles, such as biodegradable nano- or microparticles, and which have the GIT transport receptor-binding protein or derivative or analog adsorbed, coated or covalently bound, such as directly linked or linked via a linking moiety, onto the surface of the nano- or microparticle. Additionally, the protein, derivative or

analog can form the nano- or microparticle itself or the protein, derivative or analog can be covalently attached to the polymer or polymers used in the production of the biodegradable nano- or microparticles or drug-loaded or drug-  
5 encapsulated nano- or microparticles or the peptide can be directly conjugated to the active agent. Such conjugations to active agents include fusion proteins in which a DNA sequence coding for the peptide is fused in-frame to the gene or cDNA coding for a therapeutic peptide or protein such that  
10 the modified gene codes for a recombinant fusion protein.

In a preferred embodiment, the invention provides for treatment of various diseases and disorders by administration of a therapeutic compound (termed herein "Therapeutic"). Such "Therapeutics" include but are not  
15 limited to: GIT transport receptor-binding proteins, and analogs and derivatives (including fragments) thereof (e.g., as described hereinabove) that bind to GIT transport receptors, bound to an active agent of value in the treatment or prevention of a disease or disorder (preferably a  
20 mammalian, most preferably human, disease or disorder). Therapeutics also include but are not limited to nucleic acids encoding the GIT transport receptor-binding proteins, analogs, or derivatives bound to such a therapeutic or prophylactic active agent. The active agent is preferably a  
25 drug.

Any drug known in the art may be used, depending upon the disease or disorder to be treated or prevented, and the type of subject to which it is to be administered. As used herein, the term "drug" includes, without limitation,  
30 any pharmaceutically active agent. Representative drugs include, but are not limited to, peptides or proteins, hormones, analgesics, anti-migraine agents, anti-coagulant agents, anti-emetic agents, cardiovascular agents, anti-hypertensive agents, narcotic antagonists, chelating agents,  
35 anti-anginal agents, chemotherapy agents, sedatives, anti-neoplastics, prostaglandins, and antidiuretic agents. Typical drugs include peptides, proteins or hormones such as

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insulin, calcitonin, calcitonin gene regulating protein, atrial natriuretic protein, colony stimulating factor, betaseron, erythropoietin (EPO), interferons such as  $\alpha$ ,  $\beta$  or  $\gamma$  interferon, somatropin, somatotropin, somatostatin, 5 insulin-like growth factor (somatomedins), luteinizing hormone releasing hormone (LHRH), tissue plasminogen activator (TPA), growth hormone releasing hormone (GHRH), oxytocin, estradiol, growth hormones, leuprolide acetate, factor VIII, interleukins such as interleukin-2, and analogs 10 thereof; analgesics such as fentanyl, sufentanil, butorphanol, buprenorphine, levorphanol, morphine, hydromorphone, hydcodone, oxymorphone, methadone, lidocaine, bupivacaine, diclofenac, naproxen, paverin, and analogs thereof; anti-migraine agents such as heparin, hirudin, and 15 analogs thereof; anti-coagulant agents such as scopolamine, ondansetron, domperidone, etoclopramide, and analogs thereof; cardiovascular agents, anti-hypertensive agents and vasodilators such as diltiazem, clonidine, nifedipine, verapamil, isosorbide-5-mononitrate, organic nitrates, agents 20 used in treatment of heart disorders and analogs thereof; sedatives such as benzodiazepines, phenothiozines and analogs thereof; narcotic antagonists such as naltrexone, naloxone and analogs thereof; chelating agents such as deferoxamine and analogs thereof; anti-diuretic agents such as 25 desmopressin, vasopressin and analogs thereof; anti-anginal agents such as nitroglycerine and analogs thereof; anti-neoplastics such as 5-fluorouracil, bleomycin and analogs thereof; prostaglandins and analogs thereof; and chemotherapy agents such as vincristine and analogs thereof. 30 Representative drugs also include but are not limited to antisense oligonucleotides, genes, gene correcting hybrid oligonucleotides, ribozymes, aptameric oligonucleotides, triple-helix forming oligonucleotides, inhibitors of signal transduction pathways, tyrosine kinase inhibitors and DNA 35 modifying agents. Drugs that can be used also include, without limitation, systems containing gene therapeutics, including viral systems for therapeutic gene delivery such as

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adenovirus, adeno-associated virus, retroviruses, herpes simplex virus, sindbus virus, liposomes, cationic lipids, dendrimers, and enzymes. For instance, gene delivery viruses can be modified such that they express the targeting peptide  
5 on the surface so as to permit targeted gene delivery.

In a preferred embodiment, a Therapeutic is therapeutically or prophylactically administered to a human patient.

Additional descriptions and sources of Therapeutics  
10 that can be used according to the invention are found in various Sections herein.

#### **5.7. Therapeutic/Prophylactic Administration, Compositions and Formulations**

15 The invention provides methods of treatment (and prophylaxis) by administration to a subject of an effective amount of a Therapeutic of the invention. In a preferred aspect, the Therapeutic is substantially purified. The subject is preferably an animal, including but not limited to  
20 animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably a human.

As will be clear, any disease or disorder of interest amenable to therapy or prophylaxis by providing a  
25 drug *in vivo* systemically or by targeting a drug *in vivo* to the GIT (by linkage to a GIT transport-receptor binding protein, derivative or analog of the invention) can be treated or prevented by administration of a Therapeutic of the invention. Such diseases may include but are not limited  
30 to hypertension, diabetes, osteoporosis, hemophilia, anemia, cancer, migraine, and angina pectoris, to name but a few.

Any route of administration known in the art may be used, including but not limited to oral, nasal, topical, intravenous, intraperitoneal, intradermal, mucosal,  
35 intrathecal, intramuscular, etc. Preferably, administration is oral; in such an embodiment the GIT-transport binding protein, derivative or analog of the invention acts

advantageously to facilitate transport of the therapeutic active agent through the lumen of the GIT into the systemic circulation.

The present invention also provides therapeutic compositions/formulations. In a specific embodiment of the invention, a GIT transport receptor-binding peptide or motif of interest is associated with a therapeutically or prophylactically active agent, preferably a drug or drug-containing nano- or microparticle. More preferably, the active agent is a drug encapsulating or drug loaded nano- or microparticle, such as a biodegradable nano- or microparticle, in which the peptide is physically adsorbed or coated or covalently bonded, such as directly linked or linked via a linking moiety, onto the surface of the nano- or microparticle. Alternatively, the peptide can form the nano- or microparticle itself or can be directly conjugated to the active agent. Such conjugations include fusion proteins in which a DNA sequence coding for the peptide is fused in-frame to the gene or cDNA coding for a therapeutic peptide or protein, such that the modified gene codes for a recombinant fusion protein in which the "targeting" peptide is fused to the therapeutic peptide or protein and where the "targeting" peptide increases the absorption of the fusion protein from the GIT. Preferably the particles range in size from 200-600 nm.

Thus, in a specific embodiment, a GIT transport-binding protein is bound to a slow-release (controlled release) device containing a drug. In a specific embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); see also Levy et al., Science 228:190 (1985); During et al., Ann. Neurol. 25:351 (1989); Howard et al., J. Neurosurg. 71:105 (1989)).

5 The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a Therapeutic, and a pharmaceutically acceptable carrier. In a specific embodiment, the term

10 "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or

15 vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier

20 when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying

25 agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides.

30 Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W.

35 Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified

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form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient.

The Therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the Therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances.

## 6. EXAMPLES

### 25 6.1. Selection of GIT Receptor Targets

The HPT1, hPEPT1, D2H, and hSI receptors were selected for cloning as GIT receptor targets based on several criteria, including: (1) expression on surface of epithelial cells in gastro-intestinal tract (GIT); (2) expression along the length of small intestine (HPT1, hPEPT1, D2H); (3) expression locally at high concentration (hSI); (4) large putative extracellular domains facing into the lumen of the GIT; and (5) extracellular domains that permit easy access and bioadhesion by targeting particles.

35 The four recombinant receptor sites screened with the peptide libraries additionally have the following characteristics:

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|   | <u>Receptor</u> | <u>Characteristics</u>  |
|---|-----------------|---|
|   | D2H             | Transport of neutral/basic amino acids; a transport activating protein for a range of amino acid translocases |
| 5 | hSI             | Metabolism of sucrose and other sugars; represents 9% of brush border membrane protein in Jejunum             |
|   | HPT1            | di/tri peptide transporter or facilitator of peptide transport  |
|   | hPEPT1          | di/tri peptide transporter  |

10 Figures 1-4 (SEQ ID NOS:176, 178, 179, and 181, respectively) show the predicted amino acid sequences for hPEPT1, HPT1, hSI and D2H, respectively.

15 **6.2. Cloning of Extracellular Domain of  
Selected Receptor Site**

The following receptor domains were cloned and expressed as His-tag fusion proteins by standard techniques:

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|    | <u>Receptor</u>     | <u>Domain (amino acid residues)</u> |
|----|---------------------|-------------------------------------|
| 20 | hPEPT1 <sup>a</sup> | 391-571                             |
|    | HPT1 <sup>b</sup>   | 29-273                              |
|    | hSI <sup>c</sup>    | 272-667                             |
|    | D2H <sup>d</sup>    | 387-685                             |

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- <sup>a</sup> Liang et al., 1995, J. Biol. Chem. 270:6456-6463
  - <sup>b</sup> Dantzig et al., 1994, Association of Intestinal Peptide Transport with a Protein Related to the Cadherin Superfamily
  - <sup>c</sup> Chantret et al., Biochem. J. 285:915-923
  - <sup>d</sup> Bertran et al., J. Biol. Chem. 268:14842-14949

30 The receptor proteins were expressed as His-tag fusion proteins and affinity purified under denaturing conditions, using urea or guanidine HCl, utilizing the pET His-tag metal chelate affinity for Ni-NTA Agarose (Hochuli, E., Purification of recombinant proteins with metal chelate adsorbent, Genetic Engineering, Principals and Methods (J.K. Setlow, ed.), Plenum Press, NY, Vol. 12 (1990), pp. 87-98).

35

### 6.3. Phage Libraries

Three phage DC8, D38, and DC43 libraries expressing N-terminal pIII fusions in M13 were used to identify peptides that bind to the GIT receptors. The D38 and DC43 libraries  
5 which are composed of 37 and 43 random amino acid domains, respectively, have been described previously (McConnell et al., 1995, Molecular Diversity, 1:165-176). The DC8 library is similar to the other two except that the random insert is 8 amino acids long flanked on each side by a cysteine residue  
10 (i.e., CX<sub>8</sub>C).

### 6.4. Biopanning

Three rounds of biopanning on the GIT receptors were performed generally by standard methods (McConnell et  
15 al., 1995, Molecular Diversity, 1:165-176), using a mixture of the DC8 ( $1 \times 10^{10}$  pfu), D38 and DC43 ( $1 \times 10^{11}$  pfu) phage libraries. After each round of panning the percentage of phage recovered was determined. Following the first two rounds of panning, the eluted phage were amplified overnight.  
20 Phage from the third pan were plated out and 100 plaques were picked, amplified overnight and screened in an ELISA assay for binding to the relevant receptor and BSA. After data analysis, phage clones were identified which had high absorbance in the ELISA assay and/or a good ratio of binding  
25 to target compared to binding to BSA. The Insulin Degrading Enzyme (IDE) and recombinant human tissue factor (hTF) were used as irrelevant controls. Several variations of the standard panning technique, discussed below, were used. Selection or panning methods followed one of two strategies.  
30 The first strategy involved panning the mixed libraries on the specific GIT receptor adsorbed to a solid surface. The second strategy panned the libraries twice against the GIT receptor and then against Caco-2 cells (Peterson and Mooseker, 1992, J. Cell Science 102:581-600), Selection  
35 methods are reflected in the clone nomenclature as described below:

48

S designates the clone was identified by binding to the hS1 receptor domain.

D designates the clone was identified by binding to the D2H receptor domain.

5 P designates the clone was identified by binding to the PEPT1 receptor domain.

H designates the clone was identified by binding to the HPT-1 receptor domain.

Phage designated Ni are from a solid phase band GIT  
10 receptor pan that used the standard procedure with the addition of Ni-NTA Agarose (Qiagen, Chatsworth, CA). Receptor coated plates were blocked with 0.5% BSA/PBS containing 160 $\mu$ l Ni-NTA agarose and libraries were panned in the presence of 50 $\mu$ l Ni-NTA agarose. The receptor proteins  
15 were expressed as His-tag fusions. The His-tag has a high affinity for Ni-NTA Agarose. Blocking the plate and panning in the presence of Ni-NTA agarose minimized phage binding to the His-tag portion of the recombinant receptor.

Phage with the designation AX were eluted with acid  
20 and Factor Xa. Phage were first eluted by standard acid elution then Factor Xa (New England Biolabs, Beverly, MA: 1 $\mu$ g protease in 300 $\mu$ l of 20mM Tris-HCL, 100mM NaCl, 2mM CaCl<sub>2</sub>) was added to the panning plate and incubated 2 hours. Phage from both elution methods were pooled together then plated.

25 Phage with the designation AB were eluted with acid and base. Phage were eluted first by standard acid elution then 100mM triethylamine pH 12.1 was added to the panning plate for 10 minutes. Phage from both elution methods were pooled together then plated.

30 C designates panning on receptor followed by Caco-2 cells. First and second round pans were performed on the receptor and the third round pan was on snapwells of Caco-2 cells. DCX11, DCX8 and DCX33 were identified by two pans on D2H receptor, third pan on Caco-2 cells. The third round  
35 Factor Xa eluate from the Caco-2 cells was screened by ELISA on D2H, BSA and fixed Caco-2 cells. For HCA3 the first two rounds of panning were performed on the HPT-1 receptor and

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the third pan was on monolayers cultured on snapwells of  
Caco-2 cells.

Phage designated 5PAX were carried through five  
rounds of panning after which a number of phage were  
5 sequenced prior to screening by ELISA.

#### 6.5. Sequencing of Selected Phage

The amino acid sequence of phage inserts  
demonstrating a good ratio of binding to receptor domains  
10 and/or Caco-2 cells over background BSA binding were deduced  
from the nucleotide sequence obtained by sequencing  
(Sequenase®, U.S. Biochemical Corp., Cleveland, OH) both DNA  
strands of the appropriate region in the viral genome. The  
third round acid eluate was screened by ELISA on HPT-1, BSA  
15 and Caco-2 fixed cells. Phage designated 5PAX were carried  
through five rounds of panning after which a number of phages  
were sequenced prior to screening by ELISA.

One well of a 24 well plate was coated with 10  
µg/ml of GIT receptor and the plate was incubated overnight  
20 at 4°C. The plate was blocked with 0.5 BSA-PBS for one hour.  
A mixture of the DC8, D38 and DC43 phage libraries was added  
to the plate and the plate was incubated for 2 to 3 hours at  
room temperature on a rotator. After washing the well 10  
times with 1% BSA plus 0.05% Tween 20 in PBS, the well was  
25 eluted with 0.05M glycine, pH2. The phage was then eluted  
with 0.2M NaPO<sub>4</sub>. The eluted phage was titered on agar plates;  
the remaining phage was amplified overnight. The next day  
the amplified phage was added to a second coated plate and  
the panning procedure was repeated as described above. The  
30 eluted phage from the second pan as well as the amplified  
phage from the first pan was titered on agar plates.  
Following amplification overnight of the phage from the  
second pan, the panning procedure was repeated as described  
above. The phage eluted from the third pan and the amplified  
35 phage from the second pan were then titered overnight on agar  
plates. Isolated phage colonies were amplified overnight  
prior to use in an ELISA assay.

50

#### 6.6. Receptor ELISA Procedure

96 well plates were coated overnight with GIT receptor, BSA and, optionally, IDE (insulin degrading enzyme, an irrelevant His-fusion protein) or hTF. The plates were  
5 blocked for one hour with 0.5% BSA-PBS. After clarification, the amplified phage were diluted 1:100 in 1% BSA plus 0.05% Tween 20 in PBS and added to the plates. Following incubation of the plates on a rotator for 1 to 2 hours, the plates were washed 5 times with 1% BSA plus 0.05% Tween 20 in  
10 PBS. Dilute anti-M13-HRP conjugate (anti-M13 antibody linked to horse radish peroxidase (HRP)) was added to all the wells and the plate was incubated for one hour on a rotator. After the plates were washed 5 times, as described above, TMB substrate was added to the wells. The plates were read at  
15 650nm absorbance.

#### RECEPTOR ELISA RESULTS:

Below are the results of ELISA assays which assessed the binding of phage panned on the hSI receptor to  
20 microtiter plates coated with hSI and BSA. Table 1 shows the OD results as well as the ratio of hSI to BSA binding.

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Table 3 shows the OD results as well as the ratio of D2H to BSA binding.

Table 3

| PHAGE | Caco-2 | D2H   | BSA   | D2H/BSA |
|-------|--------|-------|-------|---------|
| DCX8  | 0.498  | 0.163 | 0.063 | 3       |
| DCX11 | 0.224  | 0.222 | 0.071 | 3       |
| DCX26 | 0.114  | 0.956 | 0.213 | 4       |
| DCX33 | 0.164  | 0.616 | 0.103 | 6       |
| DCX36 | 0.149  | 0.293 | 0.064 | 5       |
| DCX39 | 0.121  | 0.299 | 0.066 | 5       |
| DCX42 | 0.308  | 0.158 | 0.065 | 2       |
| DCX45 | 0.147  | 0.336 | 0.075 | 4       |
| Blank | 0.065  | 0.043 | 0.04  | 1       |

Below are the results of an ELISA which assessed the binding of phage panned on the hPEPT1 receptor to hPEPT1 and BSA. Table 4 shows the OD results as well as the ratio of hPEPT1 to BSA binding.

Table 4

| PHAGE  | hPEPT1 | BSA   | PEPT1/BSA |
|--------|--------|-------|-----------|
| PAX9   | 0.312  | 0.079 | 4         |
| PAX14  | 1.102  | 0.139 | 8         |
| PAX15  | 0.301  | 0.079 | 4         |
| PAX16  | 0.648  | 0.171 | 4         |
| PAX17  | 0.514  | 0.095 | 5         |
| PAX18  | 0.416  | 0.087 | 5         |
| PAX35  | 0.474  | 0.065 | 7         |
| PAX38  | 0.292  | 0.064 | 5         |
| PAX40  | 0.461  | 0.076 | 6         |
| PAX43  | 0.345  | 0.069 | 5         |
| PAX45  | 0.419  | 0.081 | 5         |
| PAX46  | 0.429  | 0.077 | 6         |
| P31    | 0.807  | 0.075 | 11        |
| P90    | 1.117  | 0.107 | 9         |
| 5PAX3  | 0.173  | 0.04  | 4         |
| 5PAX5  | 0.15   | 0.036 | 4         |
| 5PAX7  | 0.171  | 0.037 | 5         |
| 5PAX12 | 0.227  | 0.04  | 6         |
| Blank  | 0.102  | 0.039 | 3         |

Table 5 shows the results of an ELISA which assessed the binding of phage panned on the HPT-1 receptor to HPT-1 and BSA. The table shows the OD results as well as the ratio of HPT-1 to BSA binding.

5

Table 5

| PHAGE | HPT1  | BSA   | HPT/BSA |
|-------|-------|-------|---------|
| HAX9  | 0.382 | 0.075 | 5       |
| HAX40 | 0.991 | 0.065 | 15      |
| HAX42 | 0.32  | 0.071 | 5       |

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Table 6 shows the results of an ELISA which assessed the binding of phage panned for two rounds on the HPT-1 receptor followed by a third round pan on Caco-2 snapwells. Binding to fixed Caco-2 cells, HPT-1 and BSA was examined. The table shows the OD results as well as the ratio of HPT-1 to BSA binding.

15

Table 6

| PHAGE | Caco-2 | HPT1  | BSA   | HPT1/BSA |
|-------|--------|-------|-------|----------|
| HCA3  | 0.406  | 0.048 | 0.038 | 1        |

20

#### CELL ELISA PROCEDURE

Phage ELISA was used as described above with the following changes. Diluent and wash buffer was PBS containing 1%BSA and 0.05% Tween 20 and plates were washed five times at each wash step. Supernatant of infected bacterial cultures was diluted 1:100 and incubated with protein coated plates for 2-3 hours with mild agitation. Anti-M13 Horseradish peroxidase (HRP) conjugate (Pharmacia, Piscataway, NJ) was diluted 1:8000.

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Fixed Caco-2, C2BBel, and A431 cell plates were prepared by growing cells on tissue culture treated microtiter plates. When cells were confluent, plates were fixed with 10% formaldehyde, washed twice with PBS and stored with 0.5%BSA-PBS at -20°C. On the day of the assay, thawed

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plates were treated with PBS containing 0.1% phenylhydrazine for one hour at 37°C followed by two PBS washes and blocking for one hour with 0.5%BSA-PBS. The standard ELISA procedure was followed at this point.

- 5                      Phage which showed specificity to a GIT receptor was further characterized by ELISA on a variety of recombinant proteins. Phage which continued to exhibit GIT receptor specificity was sequenced.

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Table 7

TARGET BINDING PHAGE INSERT SEQUENCES:

|    | <u>hSI</u>    | <u>SEQ.</u><br><u>ID. NO.</u> |  |
|----|---------------|-------------------------------|--|
|    | <b>S15</b>    | 1                             | RSGAYESPDGRGGRSYVGGGGGCGNIGRKHNWGLRTASPACWD    |
|    | <b>S21</b>    | 2                             | SPRSFWPVVSRHESFGISNYLGCGYRTCISGMTKSSPIYPRHS    |
| 15 | <b>S22</b>    | 3                             | SSSSDWGGVPGKVVRERFKGRGCGISITSVLTGKPNPCPEPKAA   |
|    | <b>SNi10</b>  | 4                             | RVGQCTDSDVRRPWARS CAHQCGAGTRNSHGCITRPLRQASAH   |
|    | <b>SNi28</b>  | 5                             | SHSGGMNRAYGDVFRELDRWNATSHHTRPTPQLPRGPN         |
|    | <b>SNi34</b>  | 6                             | SPCGGSWGRFMQGG LFGGRTDGCGAHRNRTSASLEPPSSDY     |
|    | <b>SNi38</b>  | 7                             | RGAADQRRGWS ENLGLPRVGWDAIAHNSYTFTSRRPRPP       |
| 20 | <b>SNi45</b>  | 8                             | SGGEVSSWGRVNDLCARVSWTGCGTARSARTDNKGFLPKHSSLR   |
|    | <b>SNiAX2</b> | 9                             | SDSDGDHYGLRGGVRC SLRDRGCG LALSTVHAGPPSFYPKLSSP |
|    | <b>SNiAX4</b> | 10                            | RSLGNYGVTGTVDVTVLPMPGHANHLGVSSASSSDPPRR        |
|    | <b>SNiAX6</b> | 11                            | RTTTAKGCLLG SFGVLSGCSFTPTSPPPHLGYPPHSVN        |
|    | <b>SNiAX8</b> | 12                            | SPKLSSVGVMTKVTELPTEGPNAISIPISATLGPRNPLR        |
| 25 | <b>D2H</b>    |                               |  |
|    | <b>DAB3</b>   | 13                            | RWCGAELCNSVTKKFRPGWRDHANPSTHHRTPPPSQSSP        |
|    | <b>DAB7</b>   | 14                            | RWCGADDPGASRWRGNSLFGCGLRCSAAQSTPSGRIHSTSTS     |
|    | <b>DAB10</b>  | 15                            | SKSGEGGDSSRGETGWARVRSHAMTAGRFRWYNQLPSDR        |
| 30 | <b>DAB18</b>  | 16                            | RSSANNCEWKSDWMRRACIARYANSSGPARAVDTKAAP         |
|    | <b>DAB24</b>  | 17                            | SKWSWSSRWGSPQDKVEKTRAGCGGSPSSTNCHPYTFAPPPQAG   |
|    | <b>DAB30</b>  | 18                            | SGFWEFSRGLWDGENRKS VRS GCGFRGSSAQGPCVTPATIDKH  |
|    | <b>DAX15</b>  | 19                            | SESGRCRSVSRWMTTQ TQKGGCGSNVSRGSPLDPSHQTGHATT   |
|    | <b>DAX23</b>  | 20                            | REWRFAGPPLDLWAGPSLPSFNASSHPRALRTYWSQRPR        |
| 35 | <b>DAX24</b>  | 21                            | RMEDIKNSGWRDSCRWGD LRP GCGSRQWYPSNMRSSRDYPAGGH |
|    | <b>DAX27</b>  | 22                            | SHPWYRHNHGD FSGSGQSRHTPPESPHPGRPNATI           |

55

710550  
669150 046200



Table 8

DNA Sequences for Clones used in in vivo Pan

S15 (SEQ ID NO: 56)

5 TCTCACTCCTCGAGATCCGGCGCTTATGAGAGTCCGGATGGTTCGGGGGGGTCCGAGCTATG  
TGGGGGGCGGGGGTGGNTGTGGTAACATTGGTTCGGAAGCATAACCTGTGGGGGGCTGCGTAC  
CGCGTCGCCGGCCTGCTGGGACTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

S21 (SEQ ID NO: 57)

10 TCTCACTCCTCGAGTCCTCGCTCTTTCTGGCCCGTTGTGTCCCGGCATGAGTCGTTTGGGA  
TCTCTAACTATTTGGGNTGTGGTTATCGTACATGTATCTCCGGCACGATGACTAAGTCTAG  
CCCATTACCTCGGCATTTCGTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

S22 (SEQ ID NO: 58)

15 TCTCACTCCTCGAGTAGTAGCTCCGATTGGGGTGGTGTGCCTGGGAAGGTGGTTAGGGAGC  
GCTTTAAGGGGCGCGGTTGTGGTATTTCCATCACCTCCGTGCTCACTGGGAAGCCCAATCC  
GTGTCCGGAGCCTAAGGCGGCCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

SNi 10 (SEQ ID NO: 59)

TCTCACTCCTCGAGAGTTGGCCAGTGCACGGATTCTGATGTGCGGCGTCCTTGGGCCAGGT  
CTTGCGCTCATCAGGGTTGTGGTGCGGGCACTCGCAACTCGCACGGCTGCATACCCGTC  
TCTCCGCCAGGCTAGCGCTCATTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

20 SNi 28 (SEQ ID NO: 60)

TCTCACTCCTCGAGCCACTCCGGTGGTATGAATAGGGCCTACGGGGATGTGTTTAGGGAGC  
TTCGTGATCGGTGGAACGCCACTTCCCACCACACTCGCCCCACCCCTCAGCTCCCCCGTGG  
GCCTAATTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

SNi 34 (SEQ ID NO: 61)

25 TCTCACTCCTCGAGTCCGTGCGGGGGGTCTGTTGGGGGCGTTTTATGCAGGGTGGCCTTTTCG  
GCGGTAGGACTGATGGTTGTGGTGGCCATAGAAACCGCACTTCTGCGTCGTTAGAGCCCC  
GAGCAGCGACTACTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

SNi 38 (SEQ ID NO: 62)

30 TCTCACTCCTCGAGGGGCGCCGCCGATCAGCGGCGGGGGTGGTCCGAGAACTTGGGGTTGC  
CTAGGGTGGGGTGGGACGCCATCGCTCACAATAGCTATACGTTACCTCGCGCCGCCCGCG  
CCCCCCTCTAGA

SNi 45 (SEQ ID NO: 63)

35 TCTCACTCCTCGAGCGGTGGGGAGGTCAGCTCCTGGGGCCGCGTGAATGACCTCTGCGCTA  
GGGTGAGTTGGAAGTGGTTGTGGTACTGCTCGTTCCGCGCGTACCGACAACAAAGGCTTTCT  
TCCTAAGCACTCGTCACTCCGCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

SNi AX2 (SEQ ID NO: 64)

57



TCTCACTCCTCGAGTGATAGTGACGGGGATCATTATGGGCTTCGGGGGGGGGTGCGTTGTT  
CGCTTCGTGATAGGGGTGTGGTCTGGCCCTGTCCACCGTCCATGCTGGTCCCCCTCTTT  
TTACCCCAAGCTCTCCAGCCCCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**SNi AX4 (SEQ ID NO: 65)**

5 TCTCACTCCTCGAGGAGCTTGGGTAATTATGGCGTCACCGGGACTGTGGACGTGACGGTTT  
TGCCCATGCCTGGCCACGCCAACCACTTGGTGTCTCCTCCGCCTCTAGCTCTGATCCTCC  
GCGGCGCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**SNi AX6 (SEQ ID NO: 66)**

10 TCTCACTCCTCGAGAACTACGACGGCTAAGGGGTGTCTTCTCGGAAGCTTCGGCGTTCTTA  
GTGGGTGCTCATTACGCCAACCTCTCCACCGCCCCACCTAGGATACCCCCCCCCACTCCGT  
CAATTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**SNi AX8 (SEQ ID NO: 67)**

15 TCTCACTCCTCGAGCCCGAAGTTGTCCAGCGTGGGTGTTATGACTAAGGTCACGGAGCTGC  
CCACGGAGGGGCCCTAACGCCATTAGTATTCCGATCTCCGCGACCCTCGGCCGCGCAACCC  
GCTCCGCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**DAB3 (SEQ ID NO: 68)**

TCTCACTCCTCGAGGTGGTGCGGCGCTGAGCTGTGCAACTCGGTGACTAAGAAGTTTTCGCC  
CGGGCTGGCGGGATCACGCCAATCCCTCCACCCATCATCGTACTCCCCCGCCCAGCCAGTC  
CAGCCCTTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

20 **DAB7 (SEQ ID NO: 69)**

TCTCACTCCTCGAGGTGGTGCGGCGCTGATGACCCGTGTGGTGCCAGTCGTTGGCGGGGGG  
GCAACAGCTTGTGTTGGTTGTGGTCTTCGTTGTAGTGCGGCGCAGAGCACCCCGAGTGGCAG  
GATCCATTCCACTTCGACCAGCTCTAGAATCGAAGGTGCGCTAGACCTTCGAGA

**DAB10 (SEQ ID NO: 70)**

25 TCTCACTCCTCGAGTAAGTCCGGGGAGGGGGGTGACAGTAGCAGGGGGCGAGACGGGCTGGG  
CGAGGGTTTCGGTCTCACGCCATGACTGCTGGCCGCTTTCGGTGGTACAACCAGTTGCCCTC  
TGATCGGTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**DAB18 (SEQ ID NO: 71)**

30 TCTCACTCCTCGAGGTGAGCGCCAATAATTGCGAGTGGAAGTCTGATTGGATGCGCAGGG  
CCTGTATTGCTCGTTACGCCAACAGTTCGGGCCCCGCCCCGCGCCGTCGACACTAAGGCCGC  
GCCCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**DAB24 (SEQ ID NO: 72)**

35 TCTCACTCCTCGAGTAAGTGGTCGTGGAGTTCGAGGTGGGGCTCCCCGCAGGATAAGGTTG  
AGAAGACCAGGGCGGGTGTGGTGGTAGTCCCAGCAGACCAATTGTCACCCCTACACCTT  
TGCCCCCCCCCGCAAGCCGGCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

58

DAB30 (SEQ ID NO: 73)

TCTCACTCCTCGAGTGGGTTCTGGGAGTTTAGCAGGGGGCTTTGGGATGGGGAGAACCGTA  
AGAGTGTCCGGTCGGGTTGTGGTTTTTCGTGGCTCCTCTGCTCAGGGCCCGTGTCCGGTCAC  
GCCTGCCACCATTGACAAACACTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

5

DAX15 (SEQ ID NO: 74)

TCTCACTCCTCGAGTGAAGAGCGGGCGGTGCCGTAGCGTGAGCCGGTGGATGACGACGTGGC  
AGACGCAGAAGGGCGGTTGTGGTTCCAATGTTTCCCGCGGTTGCCCCCTCGACCCCTCTCA  
CCAGACCGGGCATGCCACTACTTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

10 DAX23 (SEQ ID NO: 75)

TCTCACTCCTCGAGGGAGTGGAGGTTTGCCGGGCGCCGTTGGACCTGTGGGCGGGTCCGA  
GCTTGCCCTCTTTTAACGCCAGTTCCACCCCTCGCGCCCTGCGCACCTATTGGTCCCAGCG  
GCCCCGCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

DAX24 (SEQ ID NO: 76)

15 TCTCACTCCTCGAGGATGGAGGACATCAAGAACTCGGGGTGGAGGGACTCTTGAGGTGGG  
GTGACCTGAGGCCTGGTTGTGGTAGCCGCCAGTGGTACCCCTCGAATATGCGTTCTAGCAG  
AGATTACCCCGCGGGGGGCCACTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

DAX27 (SEQ ID NO: 77)

20 TCTCACTCCTCGAGTCATCCGTGGTACAGGCATTGGAACCATGGTGACTTCTCTGGTTCGG  
GCCAGTCACGCCACACCCCGCCGGAGAGCCCCACCCCGGCCGCCCTAATGCCACCATTTC  
TAGAATCGAAGGTCGCGCTAGACCTTCGAG

DCX8 (SEQ ID NO: 78)

25 TCTCACTCCTCGAGATATAAGCACGATATCGGTTGCGATGCTGGGGTTGACAAGAAGTCGT  
CGTCTGTGCGTGGTGGTTGTGGTGCTCATTNGTCGCCACCCCGCGCCGGCCGTGGTCCTCG  
CGGCACGATGGTTAGCAGGCTTTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

DCX11 (SEQ ID NO: 79)

TCTCACTCCTCGAGTCAGGGCTCCAAGCAGTGTATGCAGTACCGCACCGGTCGTTTGACGG  
TGGGGTCTGAGTATGGTTGTGGTATGAACCCCGCCCGCCATGCCACGCCCGCTTATCCGGC  
GCGCCTGCTGCCACGCTATCGCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

30 DCX26 (SEQ ID NO: 80)

TCTCACTCCTCGAGTGGGCGGACTACTAGTGAGATTTCTGGGCTCTGGGGTTGGGGTGACG  
ACCGGAGCGGTTATGGTTGGGGTAACACGCTCCGCCCCAACTACATCCCTTATAGGCAGGC  
GACGAACAGGCATCGTTATACGTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

DCX33 (SEQ ID NO: 81)

35 TCTCACTCCTCGAGGTGGAATTGGACTGTCTTGCCCGCCACTGGCGGGCCATTACTGGACGC  
GTTTCGACGGACTATCACGCCATTAAACAATCACAGGCCGAGCATCCCCACCAGCATCCGAC  
CCCTATCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

069450: 67062060

**DCX36 (SEQ ID NO: 82)**

TCTCACTCCTCGAGTTGGTCGTCGTGGAATTGGAGCTCTAAGACTACTCGTCTGGGCGACA  
GGGCGACTCGGGAGGGTTGTGGTCCCAGCCAGTCTGATGGCTGTCCTTATAACGGCCGCCT  
TACGACCGTCAAGCCTCGCACGTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

5

**DCX39 (SEQ ID NO: 83)**

TCTCACTCCTCGAGTGGTAGTTTGAACGCATGGCAACCGCGGTCATGGGTGGGGGCGCGT  
TCCGGTCACACGCCAACAATAACTTGAACCCCAAGCCCACCATGGTTACTNGTCACCCTAC  
CTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

10 **DCX42 (SEQ ID NO: 84)**

TCTCACTCCTCGAGGTATTCGGGTTTGTCCCCGCGGGACAACGGTCCCGCTTGTAGTCAGG  
AGGCTACCTTGGAGGGTTGTGGTGCAGAGGCTGATGTCCACCCGTCGCAAGGGCCGCAA  
CTCCCCGCCCCGGGTGGACGCTCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**DCX45 (SEQ ID NO: 85)**

15 **TCTCACTCCTCGAGCGTGGGGAATGATAAGACTAGCAGGCCGGTTTCCTTCTACGGGCGCG  
T**

**PAX2 (SEQ ID NO: 86)**

20 **TCTCACTCCTCGAGTACTCCCCCAGTAGGGAGGCGTATAGTAGGCCCTATAGTGTGCGATA  
G**

**PAX9 (SEQ ID NO: 87)**

25 **TCTCACTCCTCGAGATGGCCTAGTGTGGGTTACAAGGGTAATGGCAGTGACACTATTGATG  
T**

**PAX14 (SEQ ID NO: 88)**

TCTCACTCCTCGAGAACGTTTGAAGAACGACGGGCTGGGCGTCGGCCGGTCTATTTCAGAAGA  
AGTCGGATAGGTGGTACGCCAGCCACAACATTCGTAGCCATTTTCGCGTCCATGTCTCCCGC  
TGGTAAGTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

30 **PAX15 (SEQ ID NO: 89)**

TCTCACTCCTCGAGCTATTGTCTGGGTTAAGGGTGGTGGGGAGGGGGGGGCATACGGATTCCA  
ATCTGGCTAGGTCTGGGTTGTGGTAAGGTGGCCAGGACCAGCAGGCTTCAGCATATCAACCC  
GCGCGTACCCCCCCTCCCGGTCTAGAATCGAAGGTC

**PAX16 (SEQ ID NO: 90)**

35 **TCTCACTCCTCGAGTTGGACTCGGTGGGGCAAGCACANTCATGGGGGGTTTGTGAACAAGT  
C**

60

PAX17 (SEQ ID NO: 91)

TCTCACTCCTCGAGTCAGGTTGATTTCGTTTCGTAATAGCTTTCGGTGGTATGAGCCGAGCA  
GGGCTCTGTGCCATGGTTGTGGTAAGCGCGACACCTCCACCACTCGTATCCACAATAGCCC  
CAGCGACTCCTATCCTACACGCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

5

PAX18 (SEQ ID NO: 92)

TCTCACTCCTCGAGCTTTTTGCGGTTCCAGAGTCCGAGGTTTCGAGGATTACAGTAGGACGA  
TCTNTCGGTTGCGCAACGCCACGAACCCGAGTAATGTCTCCGATGCGCACAATAACCGGGC  
CTTGGCCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

10 PAX35 (SEQ ID NO: 93)

TCTCACTCCTCGAGGAGCATCACCGACGGGGGCATCAATGAGGTGGACCTGAGTAGTGTGT  
CGAACGTTCTTGAGAACGCCAACTCGCATAGGGCCTACAGGAAGCATCGCCCGACCTTGAA  
GCGTCCTTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

PAX38 (SEQ ID NO: 94)

15 TCTCACTCCTCGAGTTTGAAGGTGAGCAGCCCGAGGGATCCGACGGTCCCGCGGAAGGGCG  
GCAATGTTGATTATGGTTGTGGTCACAGGTCTTCCGCCCCGATGCCTACCTCCGCTCTGTC  
GTGATCACGAAGTGCTACACTTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

PAX40 (SEQ ID NO: 95)

20 TCTCACTCCTCGAGAGCCAGTANGCAGGGCGGCCGGGGTGTGCCCCCTGAGTTTGGGGCGA  
GCGTTTTGGGTNGTGGTTGTGGTAGCGCCACTTATTACACGAACCTCCACCAGCTGCAAGGA  
TGCTATGGGCCACAACACTACTCGTCTAGAATCGAAGGTCGCGNTAGACCTTCGAGA

PAX43 (SEQ ID NO: 96)

TCTCACTCCTCGAGATGGTGCGAGAAGCACAAGTTTACGGCTGCGCGTTGCAGCGCGGGGG  
CGGGTTTTGAGAGGGANGCCAGCCGTCCGCCCCAGCCTGCCACCGGGGATAATACCAACCG  
TAATGCNTNTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

25

PAX45 (SEQ ID NO: 97)

TCTCACTCCTCGAGTTTTTCAGGTGTACCCGGACCATGGTCTGGAGAGGCATGCTTTGGACG  
GGACGGGTCCGCTTTACGCCATGCCCGGCCGCTGGATTAGGGCGCGTCCGCAGAACAGGGA  
CCGCCAGTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

30 PAX46 (SEQ ID NO: 98)

TCTCACTCCTCGAGCAGGTGTACGGACAACGAGCAGTGCCCCGATACCGGGANTAGGTCTC  
GTTCCGTTAGTAACGCCAGGTACTTTTCGAGCAGGTTGCTCAAGACTCACGCCCCCATCG  
CCCTTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

P31 (SEQ ID NO: 99)

35 TCTCACTCCTCGAGTGCCAGGGATAGCGGGCCTGCGGAGGATGGGTCCCGCGCCGTCCGGT  
TGAACGGGGTTGAGAACGCCAACACTAGGAAGTCTTCCCGCAGTAACCCGCGGGGTAGGCG  
CCATCCCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

61

**P90 (SEQ ID NO: 100)**

TCTCACTCCTCGAGTTCCGCCGATGCGGAGAAGTGTGCGGGCAGTCTGTTGTGGTGGGGTA  
GGCAGAACAACTCCGGTTGTGGTTCGCCCACGAAGAAGCATCTGAAGCACCGCAATCGCAG  
TCAGACCTCCTCTTCGTCCCACTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**5 5PAX3 (SEQ ID NO: 101)**

TCTCACTCCTCGAGACCGAAGAACGTGGCCGATGCTTATTCGTCTCAGGACGGGGCGGCGG  
CCGAGGAGACGTCTCACGCCAGTAATGCCGCGCGGAAGTCCCCTAAGCACAGCCCTTGAG  
GCGGCCTTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**5PAX5 (SEQ ID NO: 102)**

10 TCTCACTCCTCGAGAGGCAGTACGGGGACGGCCGGCGGCGAGCGTTCCGGGGTGCTCAACC  
TGCACACCAGGGATAACGCCAGCGGCAGCGGTTTCAAACCGTGGTACCCTTCGAATCGGGG  
TCACAAGTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**5PAX7 (SEQ ID NO: 103)**

15 TCTCACTCCTCGAGGTGGGGGTGGGAGAGGAGTCCGTCCGACTACGATTCTGATATGGACT  
TGGGGGCGAGGAGGTACGCCACCCGCACCCACCGCGCGCCCCCTCGCGTCTTGAAGGCTCC  
CCTGCCCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**5PAX12 (SEQ ID NO: 104)**

20 TCTCACTCCTCGAGGCACTGGAAGTGCGAGGGCTCTCAGGCTGCCTACGGGGACAAGGATA  
TCGGGAGGTCCAGGGGTTGTGGTTCATTACAAAGAATAACACTAATCACGCCCATCCTAG  
CCACGGCGCCGTTGCTAAGATCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**HAX9 (SEQ ID NO: 105)**

TCTCACTCCTCGAGCCGCGAGGAGGCGAACTGGGACGGCTATAAGAGGGAGATGAGCCACC  
GGAGTCGCTTTTGGGACGCCACCCACCTGTCCCGCCCTCGCCGCCCCGCTAACTCTGGTGA  
CCCTAACTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**25 HAX40 (SEQ ID NO: 106)**

TCTCACTCNTCGAGAGAGTTTCGCGGAGAGGAGGTTGTGGGGGTGTGATGACCTGAGTTGGC  
GTCTCGACGCGGAGGGTTGTGGTCCCACTCCGAGCAATCGGGCCGTCAAGCATCGCAAGCC  
CCGCCCACGCTCCCCCGCACTCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**HAX42 (SEQ ID NO: 107)**

30 TCTCACTCNTNGAGTGATCACGCGTTGGGGACGAATCTGAGGTCTGACAATGCCAAGGAGC  
CGGGTGATTACAACTGTTGTGGTAACGGGAACCTACCGGGCGAAAGGTTTTTAACCGTAG  
GCGCCCTCCGCCATCCCCANTTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

**HCA3 (SEQ ID NO: 108)**

35 TCTCACTCCTCGAGGCATATTTCTGAGTATAGCTTTGCGAATTCCCACTTGATGGGTGGCG  
AGTCCAAGCGGAAGGGTTGTGGTATTAACGGCTCCTTTTCTCCCACTTGTCGCCGCTCCCC  
CACCCAGCCTTCCGCCGCACCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

62

H40 (SEQ ID NO: 109)

TCTCACTCCTCGAGCCGGGAGAGCGGGATGTGGGGTAGTTGGTGGCGTGGTCACAGGTTGA  
ATTCCACGGGGGGTAACGCCAACATGAATGCTAGTCTGCCCCCGACCCCCCTGTTTCCAC  
TCCGTCTAGAATCGAAGGTCGCGCTAGACCTTCGAG

5 Peptide Motifs

By comparison of the amino acid sequences of the clones binding GIT receptors, certain sequence similarities or "motifs" were recognized. These motifs can often represent the part of the sequence that is important for binding to the target. Table 9 identifies regions of sequence similarity or sequence motifs (in boldface) that were identified among GIT binding peptides (corresponding SEQ ID NOS. are shown in Table 7).

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Table 9

**PEPT-1**

**HPT1**

P31

SARDSGPAEDGSRAVRLNGVENAN**TRKSSRSNPRGRRHP**

PAX9

RWPSVGKNGSDTIDVHSNDAS**TKRSLIYNHRRPLFP**

HAX42

SDHALGTNLRSDNAKEPGDYNCCGNGN**STGRK-VFNRRRPSAIP**T

PAX2

STPPSREAYSRPYSVSDSD**TNAKHSSHNRRLRTRSRPN**

20

**hSI**

SNi10

RVGQCTDSDVRRPWARS**CAHQCGAGTRNSHGCITRPLRQASAH**

SNi38

RGAADQRRG**WSEN**LGLPRVGWDAIAHNSYTFTSRRRPP

S15

RSGAYESPDGRGGRSYVGGGGCGNIGRKHN**LWGLRTAS**PACWD

SNi34

SPCGGSWGRFMQGG**LF**GGRTDGC**GAHRNRTS**ASLEPPSSDY

25

**D2H**

DAB10

SKSGEGGDSSRGETGWAR**VR**SHAMTAGRFRWYNQLPSDR

DAB30

SGFWEFSRGLWDGENRK**SV**RGCGFRG**SSA**QGPCPVTPATIDKH

DCX8

RYKHDIGCDAGVDKKSS**SV**RGCG-**AHSSPP**PRAGRGRGTMVSRL

Phage Binding to Caco-2 Cells

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Phage expressing presumed GIT binding peptide inserts were also assayed by ELISA on fixed Caco-2 or C2BBel cells as follows. Cells were plated at  $1 \times 10^5$  cells/well on 100  $\mu$ l culture media and incubated at 30°C in 5% CO<sub>2</sub> overnight. 100  $\mu$ l 25% formaldehyde was added to each well for 15 minutes. Contents of the wells were removed by inverting the plate. The plate was then washed 3 times with

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DPBS. 0.1% phenylhydrazine DPBS solution was added to each well and incubated for 1 hr at 37°C. The plate was inverted and washed 3 times. The plate was blocked with 0.5% BSA-DPBS for 1 hr at room temperature. The plate was inverted and washed 3 times with 1% BPT (PBS containing 1% BSA and 0.05% Tween20). Phage diluted with 1% BPT was added to wells containing fixed cells. Wells without phage added were used to determine background binding of the HRP conjugate. The plates were incubated 2-3 hours on a rotor at room temperature. Plates were washed as before. Plates were incubated with dilute anti-M13-HRP antibody in 1% BPT for 1 hour at room temperature. Following washing, TMB substrate was added and absorbance of the plates were read at 650 nm. Table 10 shows the relative binding of phage encoding peptides to fixed Caco-2 cells.

Table 10.

| Relative binding of phage encoding peptides to fixed Caco-2 cells |              |                                  |
|---|--------------|----------------------------------|
|   | <u>Phage</u> | <u>Fixed Caco-2 cell binding</u> |
| 25  | SNi10        | ++                               |
|   | SNi34        | +                                |
|   | P31          | ++                               |
|   | 5PAX5        | ++                               |
|   | PAX2         | +                                |
| 30  | HAX42        | +                                |
|   | DCX8         | +++                              |
|   | DCX11        | +                                |
|   | H1           | +                                |
|   | M13mpl18     | -                                |

In vivo phage selection:

Further selection of phage expressing peptides capable of binding to the GIT or transporting the GIT was done as follows. The purified library was resuspended in a

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buffer, such as TBS or PBS, and introduced onto one side of a tissue barrier, e.g., injected into the duodenum, jejunum, ileum, colon or other *in vivo* animal site using, for instance, a closed loop model or open loop model. Following  
5 injection, samples of bodily fluids located across the tissue barrier, e.g., samples of the portal circulation and/or systemic circulation, were withdrawn at predetermined time points, such as 0 to 90 minutes and/or 2 to 6 hours or more. An aliquot of the withdrawn sample (e.g., blood) was used to  
10 directly infect a host, e.g., *E. coli*, in order to confirm the presence of phage. The remaining sample was incubated, e.g., overnight incubation with *E. coli* at 37°C with shaking. The amplified phage present in the culture can be sequenced individually to determine the identity of peptides coded by  
15 the phage or, if further enrichment is desired, can be precipitated using PEG, and resuspended in PBS. The phage can then be further precipitated using PEG or used directly for administration to another animal using a closed or open GIT loop model system. Portal or systemic blood samples are  
20 collected and the phage transported into such circulation systems is subsequently amplified. In this manner, administration of the phage display library with, if desired, repeat administration of the amplified phage to the GIT of the animal, permitted the selection of phage which was  
25 transported from the GIT to the portal and/or systemic circulation of the animal.

If desired, following administration of the phage display library to the tissue barrier (e.g., GIT) of the animal model, the corresponding region of the tissue barrier  
30 can be recovered at the end of the procedures given above. This recovered tissue can be washed repeatedly in suitable buffers, e.g., PBS containing protease inhibitors and homogenized in, for example, PBS containing protease inhibitors. The homogenate can be used to infect a host,  
35 such as *E. coli*, thus permitting amplification of phages which bind tightly to the tissue barrier (e.g., intestinal tissue). Alternatively, the recovered tissue can be



homogenized in suitable PBS buffers, washed repeatedly and the phage present in the final tissue homogenate can be amplified in *E. coli*. This approach permits amplification (and subsequent identification of the associated peptides) of  
5 phages which either bind tightly to the tissue barrier (e.g., intestinal tissue) or which are internalized by the cells of the tissue barrier (e.g., epithelial cells of the intestinal tissue). This selection approach of phage which bind to tissues or which are internalized by tissues can be repeated.

10

**Treatment of animal tissue barriers  
in vivo with phage display populations**

The purified phage display library (random or preselected) was diluted to 500  $\mu$ l in PBS buffer and injected  
15 into the closed (or open) intestinal loop model (e.g., rat, rabbit or other species). At time 0 and at successive time points after injection, a sample of either the portal circulation or systemic circulation was withdrawn. An aliquot of the withdrawn blood was incubated with *E. coli*,  
20 followed by plating for phage plaques or for transduction units or for colonies where the phage codes for resistance to antibiotics such as tetracycline. The remainder of the withdrawn blood sample (up to 150  $\mu$ l) was incubated with  
25 250  $\mu$ l of *E. coli* and 5 ml of LB medium or other suitable growth medium. The *E. coli* cultures were incubated overnight by incubation at 37°C on a shaking platform. Blood samples taken at other time points (such as 15 min, 30 min, 45 min, 60 min, up to 6 hours) were processed in a similar manner, permitting amplification of phages present in the portal or  
30 systemic circulation in *E. coli* at these times. Following amplification, the amplified phage was recovered by PEG precipitation and resuspended in PBS buffer or TBS buffer. The titer of the amplified phage, before and after PEG precipitation, was determined. The amplified, PEG  
35 precipitated phage was diluted to a known phage titer (generally between  $10^8$  and  $10^{10}$  phage or plaque forming units (p.f.u.) per ml) and was injected into the GIT of the animal

closed (or open) loop model. Blood samples were collected from portal and/or systemic circulation at various time points and the phage transported into the blood samples were amplified in *E. coli* as given above for the first cycle.

5 Subsequently, the phage was PEG-precipitated, resuspended, titered, diluted and injected into the GIT of the animal closed (or open) loop model. This procedure of phage injection followed by collection of portal and/or systemic blood samples and amplification of phage transported into  
10 these blood samples can be repeated, for example, up to 10 times, to permit the selection of phages which are preferentially transported from the GIT into the portal and/or systemic circulation.

15       **6.7. Transport of Phage From Rat Lumen Into the  
          Portal and Systemic Circulation**

          Phage from random phage display libraries as well as control phage were injected into the lumen of the rat gastro-intestinal tract (*in situ* rat closed loop model).  
20 Blood was collected over time from either the systemic circulation or portal circulation and the number of phage which were transported to the circulation was determined by titering blood samples in *E. coli*.

          The phage display libraries used in this study were D38 and DC43 in which gene III codes for random 38-mer and  
25 43-mer peptides, respectively. As a negative control, the identical phage M13mp18, in which gene III does not code for a "random" peptide sequence, was used. Both the library phages D38 and DC43 were prepared from *E. coli*, mixed  
30 together, dialyzed against PBS, precipitated using PEG/NaCl and were resuspended in PBS buffer. The M13mp18 control was processed in a similar manner. The titer of each phage sample was determined and the phage samples were diluted in PBS to approximately the same titers prior to injection into  
35 the rat closed loop model.

          For sampling from the systemic circulation, approximately 15 cm of the duodenum of Wistar rats was tied

off (closed loop model), approximately 0.5ml of phage solution was injected into the closed loop and blood (0.4ml) was sampled from the tail vein at various times. The time points used (in min) were: 0, 15, 30, 45, 60, 90, 120, 180, 5 240 and 300 minutes. For sampling from the portal circulation, the portal vein was catheterized, approximately 15 cm of the duodenum was tied off (closed loop model), 0.5ml of phage solution was injected into the closed loop and blood was sampled from the portal vein catheter at various times.

10 As the portal sampling is delicate, sampling times were restricted to 15, 30, 45 and 60 minutes, where possible. The volume of phage injected into each animal was as follows:

|    | ANIMALS (15) | VOLUME OF PHAGE INJECTED |
|----|--------------|--------------------------|
| 15 | R1-R3        | 0.50 ml                  |
|    | R4           | 0.43 ml                  |
|    | R5-R15       | 0.45 ml                  |

The estimated number of transported phage has been adjusted to account for differences in volume injected into each animal (using 0.5 ml as the standard volume).

To investigate transport into the systemic circulation, animals R1, R2 and R3 received the control phage M13mp18 and animals R4, R5, R6 and R7 received the test phage D38/DC43 mix. To investigate transport into the portal circulation, animals R8, R9 and R10 received the control phage M13mp18 and animals R11, R12, R13 and R14 received the test phage D38/DC43 mix. Animal R15\* received the combined phage samples from animals R4-R7 (see Table 11) which were sampled from the systemic circulation on day one, followed by amplification in *E. coli*, PEG precipitation and resuspension in PBS. On subsequent analysis, the titer of this phage was found to be 100 times greater than the other phage samples used for animals R8-R14. Thus, the data presented for animal R15\* is adjusted down.

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Approximately 0.4 ml of the blood was collected at each time point in each model system. 30  $\mu$ l of the collected blood (systemic) was mixed with 100  $\mu$ l of the prepared *E. coli* strain K91Kan, incubated at 37°C for 30 min, and  
5 plated out for plaque formation using Top Agarose on LB plates. Various negative controls were included in the titering experiments. The following day, the number of plaque forming units was determined. Similarly, 30  $\mu$ l of the collected blood (portal) and serial dilutions (1:100, 1:1000)  
10 thereof was mixed with 100  $\mu$ l of the prepared *E. coli* strain K91Kan, incubated at 37°C for 30 min, and plated out for plaque formation using Top Agarose on LB plates. The following day, the number of plaque forming units was determined.

15 In addition, approximately 300  $\mu$ l of the collected blood from each time point (systemic and portal) was incubated with 5ml of prepared *E. coli* strain K91Kan in modified growth media containing 5mM  $MgCl_2/MgSO_4$  at 37°C overnight with shaking (to permit phage amplification). The samples were  
20 centrifuged and the cell pellet was discarded. Samples of the phage supernatant were collected, serially diluted ( $10^{-2}$ ,  $10^{-4}$ ,  $10^{-6}$ ,  $10^{-8}$ ) in TBS buffer, and plated for plaques in order to determine the number of plaque forming units present in the amplified phage samples.

25 Furthermore, an aliquot of phage was removed from the "amplified" supernatants obtained from test animals R4-R7 (samples from each time point were used), combined, and precipitated using PEG for two hours. The precipitated phage was resuspended in PBS buffer and was injected into closed  
30 loop model of animal R15\*, followed by portal sampling.

The number of phage transported from the closed loop model into the systemic circulation is presented in Table 11 hereafter. The number of phage transported from the closed loop model into the portal circulation is presented in  
35 Table 12 hereafter. These numbers are corrected for phage input difference and for volume input differences. Clearly, more phage are present in the portal samples than in the

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systemic samples, indicative of either hepatic or RES clearance and/or phage instability in the systemic circulation. In addition, the uptake of phage from the GIT into the portal circulation is quite rapid, with substantial number of phages detected within 15 minutes. The results from the portal sampling experiments would also indicate that the kinetics of uptake of phage from the D38/DC43 libraries is quicker than that of the control phage. Thus, there may be preferential uptake of phage coding for random peptide sequences from the GIT into the portal circulation. In the case of animals R13, R14 and R15\*, the % of the phage transported into the titered blood sample within the limited time frame (30, 45 and 15 mins, respectively) was estimated as 0.13%, 1.1% and 0.013%, respectively.

TABLE 11

NUMBER OF PHAGE TRANSPORTED FROM THE CLOSED LOOP MODEL INTO THE SYSTEMIC CIRCULATION

| Time (min)                        | R1  | R2 | R3  | R4 | R5  | R6 | R7 |
|-----------------------------------|-----|----|-----|----|-----|----|----|
| 0                                 | 0   | 0  | 0   | 0  | 0   | 0  | 0  |
| 15                                | 0   | 1  | 9   | 0  | 0   | 1  | 7  |
| 30                                | 2   | 1  | 0   | 0  | 46  | 1  | 11 |
| 45                                | 10  | 4  | 2   | 1  | 32  | 0  | 20 |
| 60                                | 63  | 19 | 21  | 1  | 114 | 0  | 21 |
| 90                                | 104 | 20 | 18  | 3  | 115 | 0  | 22 |
| 120                               | 94  | 24 | 27  | 0  | 64  | 0  | 6  |
| 180                               | 94  | 12 | 23  | 1  | 413 | 0  | 0  |
| 240                               | 14  | 1  | 20  | 0  | 36  | 0  | 0  |
| 300                               | 1   | 1  | 4   | 2  | 0   | 0  | 0  |
| Total number of transported phage | 382 | 83 | 124 | 8  | 820 | 2  | 87 |

Animals R1, R2 and R3 received the control phage M13mp18.

Animals R4, R5, R6 and R7 received the test phage D38/DC43 mix.

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Table 12

NUMBER OF PHAGE TRANSPORTED FROM THE CLOSED  
LOOP MODEL INTO THE PORTAL CIRCULATION

| Time<br>(min) | R8 | R9 | R10 | R11 | R12     | R13     | R14       | R15*   |
|---------------|----|----|-----|-----|---------|---------|-----------|--------|
| 15            | 15 | 6  | 3   | 1   | 19      | 231,000 | 1,000,000 | 20,000 |
| 30            | 1  | 5  | 26  | -   | 0       | 60,000  | 272,000   | -      |
| 45            | -  | 1  | 555 | -   | 1       | -       | 1,240,000 | -      |
| 60            | -  | -  | -   | -   | 420,000 | -       | -         | -      |

Animals R8, R9 and R10 received the control phage M13mp18.

Animals R11, R12, R13 and R14 received the test phage D38/DC43 mix.

Animal R15\* received the combined phage samples from animals R4-R7 (see Table 11) which were sampled from the systemic circulation on day one, followed by PEG precipitation and resuspension in PBS. On subsequent analysis, the titer of this phage was found to be 100 times greater than the other phage samples used for animals R8-R14. Thus, the data measuring phage transport into the portal circulation for animal R15\* is adjusted down.

These studies demonstrated that both the control phage and the D38/DC43 phages are transported over time from the lumen of the GIT into the portal and systemic circulation, as demonstrated by titering the phage transported to the blood in *E. coli*. More phage were transported from the test phage samples into the portal circulation than the corresponding control phage sample. In addition, the kinetics of transport of the test phage into the portal circulation appeared to exceed that of the control phage. Phage from the D38/DC43 libraries which appeared in the systemic circulation of different animals (R4-R7) were pooled, amplified in *E. coli*, precipitated, and re-applied to the lumen of the GIT, followed by collection in the portal circulation and titering in *E. coli*. These selected phage were also transported from the lumen of the GIT into the portal circulation. This *in situ* loop model may represent an



DCX39  
DCX42  
DCX45  
M13mp18

5PAX3  
5PAX5  
5PAX7  
5PAX12  
H40  
M13mp18

5

Similar to methods described herein above, these preselected phage libraries together with the negative control phage M13mp18 were injected into the rat closed loop model (6 animals per preselected phage library), blood was collected over time from the portal circulation via the portal vein and, at the termination of the experiment, a systemic blood sample was collected from the tail vein and the intestinal tissue region from the closed loop was collected.

In particular, phages selected *in vitro* to each receptor or binding site located in the GIT were amplified in *E. coli*, PEG-precipitated, resuspended in TBS and the titer of each phage sample was determined by plaquing in *E. coli* as described above. Subsequently, an equal number of each phage ( $8 \times 10^8$  phage) for each receptor site was pooled into a preselected phage library together with the negative control phage M13mp18 and each preselected phage library was administered to 6 Wistar rats per library (rats 1-6; GI-D2H, rats 7-12; GI-hSI, rats 13-18; GI-hPEPT1, and rats 19-24; GI-HPT1). Using the *in situ* loop model described above, 0.5 ml of preselected phage library solution was injected into the tied-off portion of the duodenum/jejunum. Blood was collected into heparinized tubes from the portal vein at 0, 15, 30, 45 and 60 minutes. A blood sample was taken from the systemic circulation at the end of the experiment. Similarly, the portion of the duodenum/jejunum used for phage injection was taken at the end of the experiment.

Thirty microliters of the collected portal blood (neat and  $10^{-2}$ ,  $10^{-4}$ ,  $10^{-6}$  dilutions) was added to 30  $\mu$ l *E. coli* K91Kan cells (overnight culture) and incubated at 37°C for 10 min. Subsequently, 3 ml of top agarose was added and the samples were plated for plaques. One hundred microliters of



the collected portal blood was added to 100 $\mu$ l of *E. coli* K91Kan. Five milliliters of LB medium was then added and the samples were incubated at 37°C overnight in a rotating microbial incubator. The *E. coli* was removed by  
5 centrifugation and the amplified phage supernatant samples were either titered directly or were PEG-precipitated, resuspended in TBS and titered. Following titration of the amplified phage, samples containing phage from each set of animals were combined, adjusting the titer of each sample to  
10 the same titer, and were plated for plaques on LB agar plates (22cm<sup>2</sup> square plates). Either 12,000 or 24,000 phage were plated for plaques.

Thirty microliters of the collected systemic blood (neat and 10<sup>-2</sup>, 10<sup>-4</sup>, 10<sup>-6</sup> dilutions) was added to *E. coli*  
15 K91Kan cells, incubated at 37°C for 10 min. Three ml of top agarose was then added and the samples were plated for plaques. One hundred microliters of the collected systemic blood was added to 100 $\mu$ l of *E. coli* K91Kan, incubated at 37°C for 10 min. Five milliliters of LB medium was then added and  
20 the samples were incubated at 37°C overnight in a rotating microbial incubator. The *E. coli* was removed by centrifugation and the amplified phage supernatant samples were either titered directly or were PEG-precipitated, resuspended in TBS and titered. Following titration of the  
25 amplified phage, samples containing phage from each set of animals were combined, adjusting the titer of each sample to the same titer, and were plated for plaques on LB agar plates (22cm<sup>2</sup> square plates). Either 12,000 or 24,000 phage were plated for plaques.

30 The intestinal tissue portion used in each closed loop was excised. The tissue was cut into small segments, followed by 3 washings in sterile PBS containing protease inhibitors, and homogenized in an Ultra thorex homogeniser (Int-D samples). Alternatively, the tissue (in PBS  
35 supplemented with protease inhibitors) was homogenized in an Ultra Thorex homogenizer, washed 3 times in PBS containing protease inhibitors and resuspended in PBS containing

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protease inhibitors (Int-G samples). In each case, serial dilutions (neat and  $10^{-2}$ ,  $10^{-4}$ ,  $10^{-6}$  dilutions) of the tissue homogenate was titered in *E. coli*. In addition, an aliquot (100 $\mu$ l) of the tissue homogenate was added to 100 $\mu$ l of

- 5 *E. coli* K91Kan, incubated at 37°C for 10 min, followed by addition of 5ml of LB medium and incubation overnight at 37°C in a rotating microbial incubator.

- The phage amplified from the portal blood, systemic blood and intestinal tissue was plated for plaques. The  
10 plaques were transferred to Hybond-N Nylon filters, followed by denaturation (1.5M NaCl, 0.5M NaOH), neutralization (0.5M TRIS-HCl, pH7.4, 1.5M NaCl), and washing in 2X SSC buffer. The filters were air-dried, and the DNA was cross-linked to  
15 filters were incubated in pre-hybridization buffer (6X SSC, 5X Denhardt's solution, 0.1% SDS, 20 $\mu$ g/ml yeast tRNA) at 40°C-45°C for at least 60 min.

- Synthetic oligonucleotides, (22-mers), complimentary to regions coding for the receptor or binding  
20 sites used to create the preselected phage library, were synthesized (see Table 14 below).

Table 14

OLIGONUCLEOTIDES USED IN IN VIVO SCREEN

| 25 | CLONE NAME | OLIGO                                   | SEQ.<br>ID. NO. |
|----|------------|---|-----------------|
|    | S15        | 5'TCCGGACTCTCATAAGCGCCGG <sup>3'</sup>  | 111             |
|    | S21        | 5'ACAACGGGCCAGAAAGAGCGAG <sup>3'</sup>  | 112             |
|    | S22        | 5'ACACCACCCCAATCGGAGCTAC <sup>3'</sup>  | 113             |
|    | SNi10      | 5'TCAGAATCCGTGCACTGGCCAA <sup>3'</sup>  | 114             |
| 30 | SNi28      | 5'GCCCTATTTCATACCACCGGAGT <sup>3'</sup> | 115             |
|    | SNi34      | 5'CATCAGTCCTACCGCCGAAAAG <sup>3'</sup>  | 116             |
|    | SNi38      | 5'CGTATAGCTATTGTGAGCGATG <sup>3'</sup>  | 117             |
|    | SNi45      | 5'ACGCGCGGAACGAGCAGTACCA <sup>3'</sup>  | 118             |
|    | SNiAX2     | 5'CCATAATGATCCCCGTCACTAT <sup>3'</sup>  | 119             |
| 35 | SNiAX6     | 5'AGACACCCCTTAGCCGTCGTAG <sup>3'</sup>  | 120             |
|    | SNiAX8     | 5'AGCTCCGTGACCTTAGTCATAA <sup>3'</sup>  | 121             |

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|    | CLONE NAME | OLIGO                                   | SEQ. ID. NO. |
|----|------------|---|--------------|
|    | DAB3       | 5'TGCACAGCTCAGCGCCGCACCA 3'             | 122          |
|    | DAB7       | 5'ACGGGTCATCAGCGCCGCACCA 3'             | 123          |
|    | DAB10      | 5'TGTCACCCCCCTCCCCGGA CTT 3'            | 124          |
| 5  | DAB18      | 5'ACTCGCAATTATTGGCGCTCGA 3'             | 125          |
|    | DAB24      | 5'GTCTTCTCAACCTTATCCTGCG 3'             | 126          |
|    | DAB30      | 5'AAAGCCCCCTGCTAAACTCCCA 3'             | 127          |
|    | DAX15      | 5'CTGCGTCTGCCACGTCGTCATC 3'             | 128          |
|    | DAX23      | 5'GTTAAAAGAGGGCAAGCTCGGA 3'             | 129          |
| 10 | DAX24      | 5'CCGAGTTCTTGATGTCCTCCAT 3'             | 130          |
|    | DAX27      | 5'TCCAATGCCTGTACCACGGATG 3'             | 131          |
|    | DCX8       | 5'TCGCAACCGATATCGTGCTTAT <sup>3'</sup>  | 132          |
|    | DCX11      | 5'TGCATACACTGCTTGGAGCCCT <sup>3'</sup>  | 133          |
|    | DCX26      | 5'GAAATCTCACTAGTAGTCCGCC <sup>3'</sup>  | 134          |
| 15 | DCX33      | 5'GCGGGCAAGACAGTCCAATTCC <sup>3'</sup>  | 135          |
|    | DCX36      | 5'GAGCTCCAATTCCACGACGACC <sup>3'</sup>  | 136          |
|    | DCX39      | 5'GGTTGCCATGCGTTCAAAC TAC <sup>3'</sup> | 137          |
|    | DCX42      | 5'TCCCGCGGGGACAAACCCGAAT <sup>3'</sup>  | 138          |
|    | DCX45      | 5'CTGCTAGTCTTATCATTCCCCA <sup>3'</sup>  | 139          |
| 20 | PAX2       | 5'CTATCGACACTATAGGGCCTAC <sup>3'</sup>  | 140          |
|    | PAX9       | 5'TACCCTTGTAACCCACACTAGG <sup>3'</sup>  | 141          |
|    | PAX14      | 5'TTCTTCTGAATAGACCGGCCGA <sup>3'</sup>  | 142          |
|    | PAX15      | 5'CCACCACCCTTAACCCGACAAT <sup>3'</sup>  | 143          |
|    | PAX16      | 5'AGGGGGAGACTTGTTCA CAAAC <sup>3'</sup> | 144          |
| 25 | PAX17      | 5'CGGCTCATACCACCGAAAGCTA <sup>3'</sup>  | 145          |
|    | PAX18      | 5'ATCGTCCTACTGTAATCCTCGA <sup>3'</sup>  | 146          |
|    | PAX35      | 5'GACACACTACTCAGGTCCACCT <sup>3'</sup>  | 147          |
|    | PAX38      | 5'CCATAATCAACATTGCCGCCCCT <sup>3'</sup> | 148          |
|    | PAX40      | 5'CAAAACGCTCGCCCCAAACTCA <sup>3'</sup>  | 149          |
| 30 | PAX43      | 5'GTAAACTTG TGCTTCTCGCACC <sup>3'</sup> | 150          |
|    | PAX45      | 5'CCATGGTCCGGGTACACCTGAA <sup>3'</sup>  | 151          |
|    | PAX46      | 5'GTTACTAACGGAACGAGACCTA <sup>3'</sup>  | 152          |
|    | P31        | 5'TGTTGGCGTTCTCAACCCCGTT <sup>3'</sup>  | 153          |
|    | P90        | 5'ACAACCGGAGTTGTTCTGCCTA <sup>3'</sup>  | 154          |
| 35 | 5PAX3      | 5'TAAGCATCGGCCACGTTCTTCG <sup>3'</sup>  | 155          |
|    | 5PAX5      | 5'TTATCCCTGGTGTGCAGGTTGA <sup>3'</sup>  | 156          |

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|    | CLONE NAME     | OLIGO                                    | SEQ.<br>ID. NO. |
|----|----------------|--|-----------------|
|    | 5PAX7          | 5'TATCAGAATCGTAGTCGGACGG <sup>3</sup> '  | 157             |
|    | 5PAX12         | 5'CTTTGTAATGGAACCACAACCC <sup>3</sup> '  | 158             |
|    | HAX9           | 5'CGGTGGCTCATCTCCCTCTTAT <sup>3</sup> '  | 159             |
| 5  | HAX35          | 5'ATCAGACTGGCTGGGACCACAA <sup>3</sup> '  | 160             |
|    | HAX40          | 5'CACAACCTCCTCTCCGCGAACT <sup>3</sup> '  | 161             |
|    | HAX42          | 5'AGATTCGTCCCCAACGCGTGAT <sup>3</sup> '  | 162             |
|    | HCA3           | 5'GGGAATTGCGCAAAGCTATACTC <sup>3</sup> ' | 163             |
|    | H40            | 5'CCCCGTGGAATTCAACCTGTGA <sup>3</sup> '  | 164             |
| 10 | M13 (positive) | 5'GTCGTCTTTCCAGACGT <sup>3</sup> '       | 165             |
|    | M13 (negative) | 5'CTTGCATGCCTGCAGGTCGAC <sup>3</sup> '   | 166             |

The oligonucleotides (5pmol) were 5'end labelled with <sup>32</sup>P-ATP and T4 polynucleotide kinase and approximately 2.5pmol of labelled oligonucleotide was used in hybridization studies. Hybridizations were performed at 40-45°C overnight in buffer containing 6X SSC, 5X Denhardt's solution, 0.1% SDS, 20µg/ml yeast tRNA and the radiolabeled synthetic oligonucleotide, followed by washings (20-30 min at 40-45°C) in the following buffers: (i) 2X SSC / 0.1% SDS, (ii) 1X SSC / 0.1% SDS, (iii) 0.1X SSC / 0.1% SDS. The filters were air-dried and exposed for autoradiography for 15 hours, 24 hours or 72 hours.

Hybridization data indicated that all the oligonucleotide probes bound specifically to their phage target except for the HAX9 probe which apparently was not labeled. A negative control probe that hybridized only to M13mp18 DNA showed a weak to negative signal in all samples tested (data not shown).

Hybridization data for pools from each receptor group of rats was compiled. Tables 15, 16, 17 and 18 show a representative compilation of autoradiograph signals of the HSI, D2H, HPT1 and hPEPT1 receptor groups. These Tables show the phage absorption and uptake from the closed loop GIT model to portal and systemic circulation and phage absorption/internalization to intestinal tissue. In these Tables, Int-G refers to intestinal tissue homogenized prior

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to washing and recovery while Int-D refers to intestinal tissue washed prior to homogenization and phage recovery. In all cases, leading phage candidates were present in more than one animal.

5

Table 15

SUMMARY OF AUTORADIOGRAPH SIGNALS OF HSI ANIMAL STUDY

10

| Phage   | Portal  | Int.-G | Int.-D |
|---------|---------|--------|--------|
| S15     | ++      | +/-    | +/-    |
| S21     | -       | -      | -      |
| S22     | -       | -/+    | -      |
| SNi-10  | +++ / + | ++     | ++     |
| SNi-28  | -       | -      | -      |
| SNi-34  | ++      | -      | -      |
| SNi-38  | ++      | -      | -      |
| SNi-45  | -       | -      | -      |
| SNiAX-2 | -       | -      | -      |
| SNiAX-6 | -       | -      | -      |
| SNiAX-8 | -       | -      | -      |
| M13     | ++++++  | ++++++ | ++++++ |
| M13     | nd*     | +      | -      |

15

20

\*not detected

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30

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T, 0780  
065730-07802000

Table 16

SUMMARY OF AUTORADIOGRAPH SIGNALS OF D2H ANIMAL STUDY

| Phage   | Portal | Int.-G | Int.-D |
|---------|--------|--------|--------|
| DAB3    | +++    | +/-    | -/+    |
| DAB7    | ++     | ++     | -/+    |
| DAB10   | ++++++ | +/-    | -/+    |
| DAB18   | -      | -      | -      |
| DAB24   | -      | -      | -      |
| DAB30   | ++++   | ++     | +++    |
| DAX15   | -      | -      | -      |
| DAX23   | -/+    | +      | -/+    |
| DAX24   | -      | -      | -      |
| DAX27   | -      | +      | -      |
| DCX8    | +++++  | +/-    | -      |
| DCX11   | ++++++ | ++     | -/+    |
| DCX26   | -      | -      | -      |
| DCX33   | +++    | ++     | ++     |
| DCX36   | -      | -      | -      |
| DCX39   | -      | -/+    | -      |
| DCX42   | -      | -      | -/+    |
| DCX45   | -      | ++     | -      |
| M13 (+) | +++++  | +++++  | +++++  |
| M13 (-) | +/-    | -/+    | -      |

Table 17

SUMMARY OF AUTORADIOGRAPH SIGNALS OF HPT1 ANIMAL STUDY

| Phage   | Int.-G | Portal | Systemic |
|---------|--------|--------|----------|
| H40     | -      | -      | ++++     |
| HAX9    | ND     | ND     | ND       |
| HAX35   | -      | +      | -        |
| HAX40   | -      | -      | -        |
| HAX42   | -      | ++     | ++       |
| HCA3    | -      | -      | -        |
| PAX2    | -      | +++    | ++++     |
| M13 (+) | ++++++ | ++++++ | ++++++   |
| M13 (-) | -      | --/+   | -        |

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Table 18

## SUMMARY OF AUTORADIOGRAPH SIGNALS OF hPEPT1 ANIMAL STUDY

| Phage   | Int.-G | Portal | Systemic |
|---------|--------|--------|----------|
| PAX2    | -      | ++     | -        |
| PAX9    | ++     | +++    | -        |
| PAX14   | -      | ++     | -        |
| PAX15   | -/+    | -      | -        |
| PAX16   | -      | -      | -        |
| PAX17   | +      | ++/+   | -        |
| PAX18   | -      | -      | -        |
| PAX35   | -      | -      | -        |
| PAX38   | -/+    | -      | -        |
| PAX40   | +      | +++    | -        |
| PAX43   | +      | -      | -        |
| PAX45   | -      | -      | -        |
| PAX46   | -      | +++    | -        |
| P31     | ++     | ++++   | ++       |
| 5PAX3   | ++/+   | ++     | -        |
| 5PAX5   | -      | -      | ++       |
| 5PAX7   | +++    | -      | -        |
| 5PAX12  | ++++   | ++     | -        |
| H40     | ++     | ++     | -        |
| M13 (+) | ++++++ | ++++++ | ++++++   |
| M13 (-) | -      | -      | -        |

Apart from the synthetic oligonucleotide to HAX9, all oligonucleotides were initially confirmed to be radiolabeled, as determined by hybridization to the corresponding phage target (eg., phage S15 hybridized to the oligonucleotide S15). In addition, under the experimental conditions used, the oligonucleotides essentially did not hybridize to the negative control phage template M13mp18. Two oligonucleotides were synthesized to the phage M13mp18: (1) a positive oligonucleotide which hybridizes to a conserved sequence in both M13mp18 and each of the GIT receptor or GIT binding site selected phages [designated M13 (positive)]; and (2) a negative oligonucleotide which only hybridizes to a sequence unique to the multiple cloning site of phage M13mp18 and which does not hybridize to any of the GIT receptor or GIT binding site selected phages.

In the case of the hSI pool of phages, only four phages were transported from the closed loop model into the portal circulation: phages S15, SNI-10, SNI-34 and SNI-38. The other phages, S21, S22, SNI-28, SNI-45, SNIAX-2, SNIAX-6 and 5 SNIAX-8, were not transported from the GIT into the portal circulation. In addition, phages SNI-10 and to a lesser extent phages S15 and S22 were found in the intestine samples or fractions, whereas the other phages were not. There was a very low presence (<0.1%) of the phage M13mp18 in the Int-G 10 samples. These results show that phages can be further selected from pre-selected libraries, permitting the identification of phages which are transported from the GIT closed loop into the portal circulation or phages which bind to or are internalized by intestinal tissue.

15 In the case of the D2H pool of phages, there was a rank order by which phages were transported from the GIT closed loop model into the portal circulation, with phages DCX11 and DAB10 preferably transported, followed by phages DCX8, DAB30, DAB3 and DAB7. A number of phages from this pool were not 20 transported into the portal circulation, including phages DAB18, DAB24, DAX15, DAX24, DAX27, DCX26, DCX36, DCX39, DCX42, DCX45. There is a very low level of transport of phage DAX23 from the GIT into the portal circulation. Similarly, only some of the phages were found in the intestinal samples 25 fractions, including phages DAB30, DCX33, DAB7, DCX11, DCX45 and to a much lesser extent phages DAB3, DAB10, DCX8, DCX39, DCX42. Some phages were not found in the intestinal samples, including phages DAB18, DAB24, DAX15, DAX24, DCX26, and DCX36. There was a very low presence (<0.1%) of the phage 30 M13mp18 in the Int-G samples. These results showed that phages can be further selected from pre-selected libraries, permitting the identification of phages which are transported from the GIT closed loop into the portal circulation or phages which bind to or are internalized by intestinal 35 tissue.

In the case of the HPT1 pool of phages, there was a rank order by which phages were transported from the GIT closed

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loop model into the portal or systemic circulation. Phage PAX2 (which was used at a 4X concentration relative to the other phages in this pool) followed by phage HAX42 was found in the portal and systemic circulation; phage H40 was found in the systemic circulation only. None of the phages in this pool were found in the intestine samples or fractions. Phage M13mp18 was not found in the intestine fractions or systemic circulation, with very low incidence (<0.001%) in the portal circulation. These results show that phages can be further selected from pre-selected libraries, permitting the identification of phages which are transported from the GIT closed loop into the portal and/or systemic circulation or phages which bind to or are internalized by intestinal tissue.

In the case of the hPEPT1 pool of phages, the phages PAX2 and H40 were also included in this pool. A number of phages from this pool were found in the portal circulation, including phages P31 (SEQ ID NO:43), PAX46, PAX9, H40, PAX17, PAX40, PAX2, PAX14, 5PAX3 and 5PAX12. A number of phages were not found in the portal blood including the negative control phage M13mp18, PAX15, PAX16, PAX18, PAX35, PAX38, PAX43, PAX45, P90, 5PAX5 and 5PAX7. The only phage found in the systemic circulation were phages 5PAX5 and P31 (SEQ ID NO:43). In addition, there was preferential binding of some phages to the intestine, including phages 5PAX12, 5PAX7, 5PAX3, H40, P31 (SEQ ID NO:43), PAX9, and to a lesser extent phages PAX38 and PAX15. Some phages were not found in the intestine samples, including the negative control phage M13mp18 and the phages PAX2, PAX14, PAX16, PAX18, PAX35, PAX45, PAX46, P90 and 5PAX5. These results show that phages can be further selected from pre-selected libraries, permitting the identification of phages which are transported from the GIT closed loop into the portal and/or systemic circulation or phages which bind to or are internalized by intestinal tissue.

### Further Characterization of Select Sequences

Following initial screening of the four recombinant receptor sites (hPEPT1, HPT1, D2H, hSI) of the gastrointestinal tissue, with the phage display libraries, a series of phage were isolated which showed preferential binding to the respective target receptor sites in comparison to negative control protein BSA protein and the recombinant protein recombinant human tissue factor (hTF) (which, like the recombinant receptors of the gastrointestinal tissue, contained a poly-histidine tag at its NH<sub>2</sub>-terminal end). In subsequent experiments same titers of the selected phage which bound to each target receptor site were combined into a single pool (i.e., one pool of HPT1 binding phage, one pool of hPEPT1 binding phage, one pool of D2H binding phage, and one pool of hSI binding phage). Each pool was supplemented with an equivalent titer of the negative control phage M13mp18. These phage pools were injected into a closed duodenal loop region of rat intestinal tissue and subsequently phage was harvested and recovered which was bound to and retained by the intestinal tissue and/or was absorbed from the intestinal loop into the portal and/or systemic circulation. In addition, a selection of the initial phages which bound to the target recombinant receptor site were analyzed for binding to either fixed Caco-2 cells and/or to fixed C2BBel cells. The selection of the final lead peptide sequences was based on the ability of the phage, coding for that peptide sequence (1) to bind to the target recombinant receptor site *in vitro* in preference to its binding to the negative control proteins BSA and/or hTFs, (2) to bind to rat intestinal tissue following injection into a closed duodenal loop of rat intestinal tissue in preference to the negative control phage M13mp18, (3) to be absorbed from rat intestinal tissue into either the portal and/or systemic circulation following injection into a closed duodenal loop of rat intestinal tissue in preference to the negative control phage M13mp18, and (4) to bind to either fixed Caco-2 cells or fixed C2BBel cells in phage binding

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studies in preference to the negative control phage M13mp18. Peptides were also selected with consideration to the ease of chemical synthesis.

5           **6.9. GST Fusion Proteins of GIT Targeting Peptides**  
              **Construction of GST Fusion Proteins of GI**  
              **Targeting Peptides**

              Glutathione S-transferase (GST) vectors encoding fusion proteins of GI targeting peptides were constructed in the vector pGEX4T-2 (source, Pharmacia Biotech, Piscataway, NJ). Briefly, single-strand DNA from the clones of interest were amplified by the polymerase chain reaction. The amplified DNA was then cleaved with the restriction enzymes XhoI and NotI and then ligated into SalI/NotI cleaved pGEX4T-2. Following transformation, the DNA sequence for each construct was verified by sequencing.

              For construction of the truncated versions of the GST fusion proteins, where the inserted sequence was less than 45 base pairs, overlapping oligonucleotides containing cohesive SalI and NotI termini, and encoding the sequence of interest, were annealed and then ligated directly into SalI/NotI cleaved pGEX4T-2. Following transformation, the DNA sequence for each construct was verified.

              A diagrammatic representation of the various GST fusion protein constructs that have been synthesized is indicated in Figures 5A-5C.

**Expression and Purification of GST Fusion Proteins**

*Escherichia coli* BL21 cells containing GST fusion protein constructs were grown overnight in 2X YT media containing 100 µg/ml ampicillin (2X YT/amp). Overnight cultures were diluted 1:100 in 2X YT broth (100 ml), and cells were grown to an A<sub>600</sub> of 0.5 at 30°C, induced with 1mM isopropyl-1-thio-B-D-galactopyranoside, and grown for an additional 3 h. Cells were harvested by centrifugation and resuspended in 5 ml of PBS containing a mixture of the proteinase inhibitors (Boehringer/Mannheim). Cells were

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sonicated on ice, and the cell lysates were centrifuged at 12,000 x g for 10 minutes at 4°C. Supernatant fractions were reacted for 30 minutes at room temperature with 2 ml of a 50% slurry of glutathione-Sepharose® 4B, washed 3 times with 1.5 ml of PBS (at room temperature), and the bound GST fusion proteins were eluted by reaction for 10 minutes at room temperature with 3 X 1ml of 10 mM reduced glutathione in 50 mM Tris HCl pH 8.0. Protein was quantified by the Bio-Rad protein assay followed by characterization by SDS-polyacrylamide gel electrophoresis.

#### ELISA of GST fusion peptides

The standard ELISA procedure was modified as follows. GST proteins were diluted to an appropriate concentration in PBS containing 1%BSA and 0.05% Tween20 (1%BPT), titered and incubated one hour at room temperature. Following five washes an anti-GST monoclonal antibody was added (Sigma, St. Louis Clone GST-2 diluted 1:10,000 in 1%BPT) and incubated one hour. After five more washes goat anti-mouse IgG2b-HRP was added (Southern Biotechnology Associates Inc., Birmingham, AL, diluted 1:4000 in 1%BPT) and incubated one hour. After five washes plates were developed with TMB peroxidase substrate (Kirkegard and Perry, Gaithersburg, MD). All data is presented with background binding subtracted.

Figure 6 shows the binding of GST-SNi10, GST-SNi34 and GST alone to the hSI receptor and to fixed C2BBel cells.

#### GST Fusion Proteins of Selected GIT Targeting Peptides

Results show that GST-DXB8, GST-PAX2, GST-P31, GST-SNi10 and GST-SNi34 bound fixed Caco-2 or C2BBel cells (Figures 7 and 8) relative to GST control binding. GST-HAX42, GST-5PAX5, all showed weak to moderate binding relative to GST control.

Interestingly, P31 truncation 103-GST fusion protein bound almost as well as full-length P31 (SEQ ID NO:43) to fixed Caco-2 cells (A). This suggests the portion

of the P31 sequence (SEQ ID NO:43) responsible for binding resides in this portion. PAX2.107 bound similarly to full-length PAX2; therefore, this portion most likely contains the amino acid sequence responsible for binding (B). In

5 preliminary assays, none of the DCX8 truncations bound similarly to full-length DCX8 to Caco-2 cells suggesting the binding region spans more than one of these pieces.

### Inhibition of Binding by Synthetic Peptides

#### 10 Binding of GST-P31 to fixed C2BBel Cells

The standard ELISA procedure was modified as follows. GST fusion proteins and peptides were diluted to an appropriate concentration in PBS containing 1% BSA and 0.05% Tween 20. Peptides were titered, a constant concentration of  
15 diluted GST protein was added to titered peptides and the mixture was incubated one hour at room temperature. Following five washes, an anti-GST monoclonal antibody was added (Sigma, St. Louis Clone GST-2 diluted 1:10,000 in 1% BPT) and incubated one hour. After five more washes goat  
20 anti-mouse IgG2b-HRP was added (Southern Biotechnology Associates Inc., Birmingham, AL, diluted 1:4000 in 1% BPT) and incubated one hour. After five washes plates were developed with TMB peroxidase substrate (Kirkegard and Perry, Gaithersburg, MD). All data is presented with background  
25 binding subtracted.

Figures 9A and 9B show the inhibition of GST-P31 binding to C2BBel fixed cells. The peptide competitors are ZElan024 which is the dansylated peptide version of P31 (SEQ ID NO:43) and ZElan044, ZElan049 and ZElan050 which are  
30 truncated, dansylated pieces of P31 (SEQ ID NO:43). Data is presented as O.D. vs. peptide concentration and as percent inhibition of GST-P31 binding vs. peptide concentration. Uncompeted GST-P31 binding was considered as 100% binding. IC<sub>50</sub> values are estimates using the 50% line on the percent  
35 inhibition graph.

GST-P31 and GST-PAX2 exhibited no crossreactive binding to ZElan024 (P31) (SEQ ID NO:43) and ZElan018 (PAX2)

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at the 0.5  $\mu$ g/ml concentration used in competition assays. GST-HAX42 exhibited crossreactivity to ZElan018 (PAX2) and ZElan021 (HAX42) at the 5  $\mu$ g/ml concentration used in competition assays.

5                Figures 10A-10C present a compilation of data generated by competition ELISA of GST-P31, GST-PAX2, GST-SNi10 and GST-HAX42 versus various dansylated peptides on fixed C2BBe1 cells. IC<sub>50</sub> values are in  $\mu$ M and include ranges determined from multiple assays. The GST/C2BBe1 column is a  
10 summary of GST protein binding to fixed C2BBe1 cells.

#### Binding to fixed Caco-2 Cells

Caco-2 cells were fixed, treated with phenylhydrazine and blocked as described above. Synthetic  
15 peptides (100 $\mu$ g/ml) were applied in duplicate to Caco-2 cells and serially diluted down the 96-well plate. The corresponding GST-peptide fusion protein (10 $\mu$ g) was added to each well and the plates were incubated for 2h at room temperature with agitation. Binding of the GST-peptide  
20 fusion proteins to the cells was assayed using the ELISA technique described above. GST-P31 binding was inhibited by ZElan024, ZElan028 and ZElan031 as well as the two D forms ZElan053 and ZElan054. GST-PAX2 binding was inhibited by ZElan032, ZElan033, and ZElan035. GST-HAX42 binding was not  
25 inhibited by ZElan021 (full length HAX42) but it was inhibited by ZElan018 (PAX2) and ZElan026 and ZElan038 (scrambled PAX2 peptides).

#### Transport and Uptake of GST-Peptide Fusions into Live Caco-2 Cells

30                Transport and uptake of GST-peptide fusions and deletion derivatives across cultured polarized Caco-2 monolayers over 4 hours in HBSS buffer was examined using an anti-GST ELISA assay. In another experiment, transport and  
35 uptake of GST-peptide fusions and deletion derivatives across

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cultured polarized Caco-2 monolayers over 24 hours in serum-free medium (SFM) was examined using an anti-GST ELISA assay.

### Materials

5                   Buffered Hank's balanced salt solution (bHBSS) = 1x HBSS (Gibco CN.14065-031) supplemented with 0.011M glucose (1g/l), 25 mM Hepes (15 mM acid (3.575g/l; Sigma CN.H3375); 10mM base (2.603g/l; Sigma CN.H1016)].

Chloroquine: Made up as 10mM solution in water

10 [Sigma CN C6628]

Lysate buffer: 30 mM Tris-HCl pH8.0; 1mM EDTA

Serum-free medium (SFM) is normal medium without serum.

### 15 Method

a) 4h HBSS study: Transepithelial electrical flux (TER) across the Caco-2 monolayers grown on snapwells (passage 33; 23 days old) was measured to confirm monolayer integrity before beginning the experiment. The medium was  
20 removed and the cells were washed once with bHBSS. bHBSS containing 100µM chloroquine was added and the cells were incubated for 2h at 37°C. The bHBSS+chloroquine was replaced with 0.5ml bHBSS containing GST-peptide fusions (100µg/ml) and the cells were incubated as before. Basolateral samples  
25 were removed at the following times: 0, 0.5h, 2h, and 4h. At 4h, TER was measured, the apical medium was sampled and the apical reservoir was washed 6 times with HBSS. The cells were allowed to lyse for 1h on ice in lysate buffer, after which, lysate sample was collected. All samples were stored  
30 at -70°C until assay by anti-GST ELISA. Before analysis, samples were normalized for protein content relative to each other using a BioRad protein assay.

b) 24h SFM study: Transepithelial electrical flux (TER) across the Caco-2 monolayers grown on snapwells  
35 (passage 33; 23 days old) was measured to confirm monolayer integrity before beginning the experiment. The medium was removed and the cells were washed once with SFM. SFM

containing GST-peptide fusions (100µg/ml) was added to the cells which were incubated at 37°C for 24h at 5% CO<sub>2</sub>. After 24 hours, TER readings were taken, and samples from the basolateral and apical reservoirs were removed. The apical  
5 reservoir was washed 6 times with PBS. The cells were allowed to lyse for 1h on ice in lysate buffer, after which lysate sample was collected. All samples were stored at -70° until assay by anti-GST ELISA. Before analysis, samples were normalized for protein content relative to each other using a  
10 BioRad protein assay.

### **Results**

All of the GST-peptide fusions and controls examined were transported across live Caco-2 monolayers.  
15 Full-length GST-P31 and GST-DCX8, but not truncations of these molecules had a higher flux than GST alone.

Internalization of GST-peptide fusions into polarized Caco-2 cells was investigated in two experiments. In experiment 1, 15µg of GST-peptide fusion was applied in  
20 bHBSS and internalized GST-peptide was recovered by lysing the cells after 4h. In experiment 2, 10µg of GST-peptide was applied in either a) bHBSS (lysate recovered after 4h), or b) serum-free medium (lysate recovered after 24h).

Figure 11A describes complete transport of GST-  
25 peptide across a polarized Caco-2 monolayer and does not necessarily refer to internalization, *i.e.*, the GST-peptide was recovered from the basolateral reservoir of a snapwell but the proteins could have crossed the barrier by the paracellular route.

30

### **Effect of Thrombin Cleavage on Binding of GST-Peptide Fusions to Fixed Caco-2 Cells**

Binding of intact and thrombin-cleaved GST-peptide fusions to fixed Caco-2 cells was compared. Reduced binding  
35 of the thrombin-cleaved GST-peptide fusions relative to intact fusions indicates that the peptide component of the fusion, and not the GST domain, mediates binding.



## Method

Confluent Caco-2 monolayers grown in 96-well plates (p38) were fixed and treated with 0.1% phenylhydrazine before blocking with 0.1% BSA in PBS. Thirty micrograms of each GST-peptide was treated with bovine thrombin (1 $\mu$ /ml; 0.4 NIH units; Sigma CN.T9681) for 18h at room temperature in 20mM Tris-HCl pH8.0, 150mM NaCl, 2.5mM CaCl<sub>2</sub>. Controls were similarly treated without addition of thrombin. Ten micrograms of each GST-peptide fusion was removed for PAGE analysis, and 10 $\mu$ g of fusions were added in duplicate to the fixed Caco-2 cells before 5-fold serial dilutions (1% BPT diluent). The fusions were allowed to bind for 1h at room temperature. Following 6 washes with 1% BPT, binding was assayed by ELISA.

## Results

Results are shown in Figure 12.

## Conclusions:

PAGE analysis confirmed that the GST-peptide fusions were effectively cleaved with thrombin. Cleavage with thrombin significantly reduced detection of binding of GST-P31.103, GST-PAX2.106, GST-DCX8, GST-SNi10 to fixed Caco-2 cells, indicating that the peptide component, and not the GST domain, mediates binding.

### 6.10. Synthesis of Peptides

#### 6.10.1. Procedure For Solid Phase Synthesis

Peptides may be prepared by methods that are known in the art. For example, in brief, solid phase peptide synthesis consists of coupling the carboxyl group of the C-terminal amino acid to a resin and successively adding N-alpha protected amino acids. The protecting groups may be any known in the art. Before each new amino acid is added to the growing chain, the protecting group of the previous amino acid added to the chain is removed. The coupling of amino acids to appropriate resins is described by Rivier et al.,

U.S. Patent No. 4,244,946. Such solid phase syntheses have been described, for example, by Merrifield, 1964, J. Am. Chem. Soc. 85:2149; Vale et al., 1981, Science 213:1394-1397; Marki et al., 1981, J. Am. Chem. Soc. 103:3178 and in U.S.

- 5 Patent Nos. 4,305,872 and 4,316,891. In a preferred aspect, an automated peptide synthesizer is employed.

By way of example but not limitation, peptides can be synthesized on an Applied Biosystems Inc. ("ABI") model 431A automated peptide synthesizer using the "Fastmoc"

- 10 synthesis protocol supplied by ABI, which uses 2-(1H-Benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate ("HBTU") (R. Knorr et al., 1989, Tet. Lett., 30:1927) as coupling agent. Syntheses can be carried out on 0.25 mmol of commercially available
- 15 4-(2',4'-dimethoxyphenyl-(9-fluorenyl-methoxycarbonyl)-aminomethyl)-phenoxy polystyrene resin ("Rink resin" from Advanced ChemTech) (H. Rink, 1987, Tet. Lett. 28:3787). Fmoc amino acids (1 mmol) are coupled according to the Fastmoc protocol. The following side chain
- 20 protected Fmoc amino acid derivatives are used:  
FmocArg(Pmc)OH; FmocAsn(Mbh)OH; FmocAsp(<sup>t</sup>Bu)OH;  
FmocCys(Acm)OH; FmocGlu(<sup>t</sup>Bu)OH; FmocGln(Mbh)OH; FmocHis(Tr)OH;  
FmocLys(Boc)OH; FmocSer(<sup>t</sup>Bu)OH; FmocThr(<sup>t</sup>Bu)OH;  
FmocTyr(<sup>t</sup>Bu)OH. [Abbreviations: Acm, acetamidomethyl; Boc, .
- 25 tert-butoxycarbonyl; <sup>t</sup>Bu, tert-butyl; Fmoc, 9-fluorenylmethoxycarbonyl; Mbh, 4,4'-dimethoxybenzhydryl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Tr, trityl].

Synthesis is carried out using N-methylpyrrolidone (NMP) as solvent, with HBTU dissolved in

- 30 N,N-dimethylformamide (DMF). Deprotection of the Fmoc group is effected using approximately 20% piperidine in NMP. At the end of each synthesis the amount of peptide present is assayed by ultraviolet spectroscopy. A sample of dry peptide resin (about 3-10 mg) is weighed, then 20% piperidine in DMA
- 35 (10 ml) is added. After 30 min sonication, the UV (ultraviolet) absorbance of the dibenzofulvene-piperidine adduct (formed by cleavage of the N-terminal Fmoc group) is

a /

recorded at 301 nm. Peptide substitution (in mmol g<sup>-1</sup>) can be calculated according to the equation:

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$$\text{substitution} = \frac{A \times v}{7800 \times w} \times 1000$$

5 where A is the absorbance at 301 nm, v is the volume of 20% piperidine in DMA (in ml), 7800 is the extinction coefficient (in mol<sup>-1</sup>dm<sup>3</sup>cm<sup>-1</sup>) of the dibenzofulvene-piperidine adduct, and w is the weight of the peptide-resin sample (in mg).

10 Finally, the N-terminal Fmoc group is cleaved using 20% piperidine in DMA, then acetylated using acetic anhydride and pyridine in DMA. The peptide resin is thoroughly washed with DMA, CH<sub>2</sub>Cl<sub>2</sub> and finally diethyl ether.

#### 15 6.10.2. Cleavage and Deprotection

By way of example but not limitation, cleavage and deprotection can be carried out as follows: The air-dried peptide resin is treated with ethylmethyl-sulfide (EtSMe), ethanedithiol (EDT), and thioanisole (PhSMe) for approximately 20 min. prior to addition of 95% aqueous trifluoroacetic acid (TFA). A total volume of approximately 50 ml of these reagents are used per gram of peptide-resin. The following ratio is used: TFA:EtSMe:EDT:PhSMe (10:0.5:0.5:0.5). The mixture is stirred for 3 h at room temperature under an atmosphere of N<sub>2</sub>. The mixture is filtered and the resin washed with TFA (2 x 3 ml). The combined filtrate is evaporated *in vacuo*, and anhydrous diethyl ether added to the yellow/orange residue. The resulting white precipitate is isolated by filtration. See King et al., 1990, Int. J. Peptide Protein Res. 36:255-266 regarding various cleavage methods.

#### 35 6.10.3. Purification of the Peptides

Purification of the synthesized peptides can be carried out by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography, high performance liquid chromatography

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(HPLC)), centrifugation, differential solubility, or by any other standard technique.

**6.10.4. Conjugation of Peptides  
to Other Molecules**

The peptides of the present invention may be linked to other molecules (e.g., a detectable label, a molecule facilitating adsorption to a solid substratum, or a toxin, according to various embodiments of the invention) by methods that are well known in the art. Such methods include the use of homobifunctional and heterobifunctional cross-linking molecules.

The homobifunctional molecules have at least two reactive functional groups, which are the same. The reactive functional groups on a homobifunctional molecule include, for example, aldehyde groups and active ester groups. Homobifunctional molecules having aldehyde groups include, for example, glutaraldehyde and subaraldehyde. The use of glutaraldehyde as a cross-linking agent was disclosed by Poznansky et al., 1984, Science 223:1304-1306.

Homobifunctional molecules having at least two active ester units include esters of dicarboxylic acids and N-hydroxysuccinimide. Some examples of such N-succinimidyl esters include disuccinimidyl suberate and dithio-bis-(succinimidyl propionate), and their soluble bis-sulfonic acid and bis-sulfonate salts such as their sodium and potassium salts. These homobifunctional reagents are available from Pierce, Rockford, Illinois.

The heterobifunctional molecules have at least two different reactive groups. Some examples of heterobifunctional reagents containing reactive disulfide bonds include N-succinimidyl 3-(2-pyridyl-dithio)propionate (Carlsson et al., 1978, Biochem J. 173:723-737), sodium S-4-succinimidyloxycarbonyl-alpha-methylbenzylthiosulfate, and 4-succinimidyloxycarbonyl-alpha-methyl-(2-pyridyldithio)toluene. N-succinimidyl 3-(2-pyridyldithio)propionate is preferred. Some examples of

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heterobifunctional reagents comprising reactive groups having a double bond that reacts with a thiol group include succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate and succinimidyl m-maleimidobenzoate.

5 Other heterobifunctional molecules include succinimidyl 3-(maleimido)propionate, sulfosuccinimidyl 4-(p-maleimido-phenyl)butyrate, sulfosuccinimidyl 4-(N-maleimidomethyl-cyclohexane)-1-carboxylate, maleimidobenzoyl-N-hydroxy-succinimide ester. The sodium sulfonate salt of  
10 succinimidyl m-maleimidobenzoate is preferred. Many of the above-mentioned heterobifunctional reagents and their sulfonate salts are available from Pierce.

Additional information regarding how to make and use these as well as other polyfunctional reagents may be  
15 obtained from the following publications or others available in the art: Carlsson et al., 1978, Biochem. J. 173:723-737; Cumber et al., 1985, Methods in Enzymology 112:207-224; Jue et al., 1978, Biochem 17:5399-5405; Sun et al., 1974, Biochem. 13:2334-2340; Blattler et al., 1985, Biochem.  
20 24:1517-152; Liu et al., 1979, Biochem. 18:690-697; Youle and Neville, 1980, Proc. Natl. Acad. Sci. USA 77:5483-5486; Lerner et al., 1981, Proc. Natl. Acad. Sci. USA 78:3403-3407; Jung and Moroi, 1983, Biochem. Biophys. Acta 761:162; Caulfield et al., 1984, Biochem. 81:7772-7776; Staros, 1982,  
25 Biochem. 21:3950-3955; Yoshitake et al., 1979, Eur. J. Biochem. 101:395-399; Yoshitake et al., 1982, J. Biochem. 92:1413-1424; Pilch and Czech, 1979, J. Biol. Chem. 254:3375-3381; Novick et al., 1987, J. Biol. Chem. 262:8483-8487; Lomant and Fairbanks, 1976, J. Mol. Biol. 104:243-261; Hamada  
30 and Tsuruo, 1987, Anal. Biochem. 160:483-488; Hashida et al., 1984, J. Applied Biochem. 6:56-63.

Additionally, methods of cross-linking are reviewed by Means and Feeney, 1990, Bioconjugate Chem. 1:2-12.

#### 35 6.10.4.1. Biotinylation of Peptides

Methods of biotinylating peptides are well known in the art. Any convenient method may be employed in the

practice of the invention. For example, the following procedure was used. Ten micrograms of peptide was dissolved in 100  $\mu$ l of 0.1 % acetic acid. PBS (900 $\mu$ l) and 3.3 mg of biotin-LC-NHS (Pierce, Rockford, IL) was added. Following 5 incubation for 30 minutes at room temperature the biotinylated peptides were purified over a Superose 12 column (Pharmacia, Piscataway, NJ).

#### 6.10.5. Synthetic Peptides

10 Tables 19, 20 and 21 provide the primary structure for various synthetic peptides manufactured in the practice of the present invention.

00070849-054599  
1256011

| Table 19  |                  |  |
|-----------|------------------|--|
| Seq ID No | Peptide name     | Sequence   |
|           | ELAN005          | H <sub>2</sub> N-C-K(dns) -<br>FITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQ-CONH <sub>2</sub> |
| 20        | ELAN006          | Ac-CLNGGVKMYVESVDRYVC-CONH <sub>2</sub>  |
|           | FITC-<br>ELAN006 | Ac-CLNGGVK (FITC) MYVESVDRYVC-CONH <sub>2</sub>                                      |
|           | ELAN006ii        | H <sub>2</sub> N-C-K(dns) -RLNGGVSMYVESVDRYVCR-CONH <sub>2</sub>                     |
| 167       | ELAN007          | H <sub>2</sub> N-RIAGLPWYRCRTVAFETGMQNTQLCSTIVQLSFTPEE-COOH                          |
| 193       | ELAN007ii        | H <sub>2</sub> N-KKRIAGLPWYRCRTVAFETGMQNTQLCSTIVQLSFTPEE-CONH <sub>2</sub>           |
| 25        | bZElan008 (P31)  | biotin-K(dns) SARDSGPAEDGSRAVRLNGVENANTRKSSR SNPRGRRHP-COOH                          |
|           | bZElan009        | biotin-K(dns) SSADAEKCAGSLLWWGRQNNSGCGSPTKKH LKHRNRSQTSSSSHG-COOH                    |
| 168       | ELAN010          | H <sub>2</sub> N-REFAERRLWGCDLDSWRLDAEGCGPTPSNRAVKHRKPRPR SPAL-COOH                  |
|           | bZElan010        | biotin-K(dns) REFAERRLWGCDLDSWRLDAEGCGPTPSNR AVKHRKPRPRSPAL-COOH                     |
| 30        | 169              | ELAN012  |
|           |                  | H <sub>2</sub> N-SGSHSGGMNRAYGDVFRELDRWYATSHHTRPTPQLPRGPN-COOH                       |
|           | bELAN012         | biotin-SGSHSGGMNRAYGDVFRELDRWYATSHHTRPTPQLPRGPN-COOH                                 |
| 35        | ZElan012         | H <sub>2</sub> N-K(dns) SGSHSGGMNRAYGDVFRELDRWYATSHHTRPTPQLP RGPN-COOH               |

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|     |                            |  |  |
|-----|----------------------------|--|--|
| 249 | ELAN013                    | H <sub>2</sub> N-<br>SGSPPCGGSWGRFMQGGLFGGRTDGCGAHRNRTSASLEPPSSD<br>Y-CONH <sub>2</sub>          |  |
| 250 | ELAN014                    | H <sub>2</sub> N-<br>SHSGGMNRAYGDVFRELDRWNATSHHTRPTPQLPRGPNS-<br>CONH <sub>2</sub>               |  |
| 5   | bZElan014                  | biotin-<br>K (dns) SHSGGMNRAYGDVFRELDRWNATSHHTRPTPQLPRG<br>PNS-CONH <sub>2</sub>                 |  |
|     | ZElan014                   | H <sub>2</sub> N-<br>K (dns) SHSGGMNRAYGDVFRELDRWNATSHHTRPTPQLPRG<br>PNS-CONH <sub>2</sub>       |  |
| 10  | ZElan015<br>(DCX11)        | H <sub>2</sub> N-<br>K (dns) SQGSKQCMQYRTGRLTVGSEYGCGMNPARGHATPAYPA<br>RLLPRYR-CONH <sub>2</sub> |  |
|     | ZElan016<br>(SNI10)        | H <sub>2</sub> N-<br>K (dns) RVGQCTDSDVRRPWARSACAHQGCAGTRNSHGCITRP<br>LRQASAH-CONH <sub>2</sub>  |  |
|     | bZElan017                  | biotin-K (dns) SGSGRVGQCTDSDVRRPWARSAC-CONH <sub>2</sub>   |  |
|     | ZElan017                   | H <sub>2</sub> N-K (dns) RVGQCTDSDVRRPWARSAC-CONH <sub>2</sub>                                   |  |
| 15  | ZElan018<br>(PAX2)         | H <sub>2</sub> N-<br>K (dns) STPPSREAYSRPYSVDSDDTNAKHSSHNRLRTRSR<br>PNG-CONH <sub>2</sub>        |  |
|     | ZElan019<br>(5PAX5)        | H <sub>2</sub> N-<br>K (dns) RGSTGTAGGERSGVLNLHTRDNASGSGFKPWYPSNRG<br>HK-CONH <sub>2</sub>       |  |
|     | ZElan020<br>(CY09)         | H <sub>2</sub> N-K (dns) SGSGLYANPGMYSLHSPA-CONH <sub>2</sub>                                    |  |
| 20  | bZElan020<br>(CY09)        | biotin-K (dns) SGSGLYANPGMYSLHSPA-CONH <sub>2</sub>  |  |
|     | ZElan021<br>(HAX42)        | H <sub>2</sub> N-<br>K (dns) SDHALGTNLRSDNAKEPGDYNCCGNGNSTGRKVFNR<br>RPSAIP-CONH <sub>2</sub>    |  |
|     | ZElan022<br>(SNI34)        | H <sub>2</sub> N-<br>K (dns) SPCGGSWGRFMQGGLFGGRTDGCGAHRNRTSASLEPP<br>SSDY-CONH <sub>2</sub>     |  |
| 25  | ZElan023<br>(DCX8)         | H <sub>2</sub> N-<br>K (dns) RYKHDIGCDAGVDKKSSSVRGCGAHSSPPRAGRGP<br>RGTMSRL-CONH <sub>2</sub>    |  |
|     | ZElan024<br>(P31)          | H <sub>2</sub> N-<br>K (dns) SARDSGPAEDGSRAVRLNGVENANTRKSSRSNPRGR<br>RHPGG-CONH <sub>2</sub>     |  |
| 30  | ZElan025<br>(DAB10)        | H <sub>2</sub> N-<br>K (dns) SKSGEGGDSSRGETGWARVRSHAMTAGRFRWYNQLPS<br>DR-CONH <sub>2</sub>       |  |
|     | ZElan026<br>(PAX2/control) | H <sub>2</sub> N-<br>K (dns) SEANLDGRKSRYSPPRNSSTRPRTSPNSVHARYPST<br>DHD-CONH <sub>2</sub>       |  |
|     | bELAN027<br>(PAX2)         | biotin-<br>SGSGSTPPSREAYSRPYSVDSDDTNAKHSSHNRLRTRSRPN<br>G-CONH <sub>2</sub>                      |  |
| 35  | 251                        | 18C21<br>Fmoc-<br>Z16N23   | H <sub>2</sub> N-DTNAKHSSHNRLRTRSRPNG-CONH <sub>2</sub><br>Fmoc-K (dns) RVGQCTDSDVRRPWARSACAHQG-COOH |
|     | 252                        | 16C23  | H <sub>2</sub> N-CGAGTRNSHGCITRPLRQASAHG-CONH <sub>2</sub>   |

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|    |           |   |
|----|-----------|---|
|    | Z16C23    | H <sub>2</sub> N-K (dns) CGAGTRNSHGICITRPLRQASAHG-CONH <sub>2</sub> |
|    | ZElan028  | H <sub>2</sub> N-K (dns) ENANTRKSSRSNPRGRRHPG-CONH <sub>2</sub>     |
|    | (P31      |   |
|    | fragment) |   |
| 5  | ZElan029  | H <sub>2</sub> N-K (dns) TRKSSRSNPRG-CONH <sub>2</sub>              |
|    | (P31      |   |
|    | fragment) |   |
|    | ZElan030  | H <sub>2</sub> N-K (dns) ENANTRKSSRSNPRG-CONH <sub>2</sub>          |
|    | (P31      |   |
|    | fragment) |   |
|    | ZElan031  | H <sub>2</sub> N-K (dns) TRKSSRSNPRGRRHPG-CONH <sub>2</sub>         |
|    | (P31      |   |
|    | fragment) |   |
| 10 | ZElan032  | H <sub>2</sub> N-K (dns) TNAKHSSHNRRLRTRSRPN-CONH <sub>2</sub>      |
|    | (PAX2     |   |
|    | fragment) |   |
|    | ZElan033  | H <sub>2</sub> N-K (dns) TNAKHSSHNRRLRTR-CONH <sub>2</sub>          |
|    | (PAX2     |   |
|    | fragment) |   |
|    | ZElan034  | H <sub>2</sub> N-K (dns) SSHNRRLRTRSRPN-CONH <sub>2</sub>           |
|    | (PAX2     |   |
| 15 | fragment) |   |
|    | ZElan035  | H <sub>2</sub> N-K (dns) SSHNRRLRTR-CONH <sub>2</sub>               |
|    | (PAX2     |   |
|    | fragment) |   |
|    | ZElan036  | H <sub>2</sub> N-K (dns) VRRPWARSCAHQCGAGTRNS-CONH <sub>2</sub>     |
|    | (SNI10    |   |
|    | fragment) |   |
| 20 | ZElan037  | H <sub>2</sub> N-K (dns) CTDSVRRPWARSC-CONH <sub>2</sub>            |
|    | (SNI10    |   |
|    | fragment) |   |
|    | ZElan038  | H <sub>2</sub> N-   |
|    | (PAX2/con | K (dns) SRANTDGRKSRYSPPRRNSSTEPRLSPNSVHARYPST                       |
|    | trol)     | DHD-CONH <sub>2</sub>   |
|    | ZElan039  | H <sub>2</sub> N-K (dns) ENANTRKSSR-CONH <sub>2</sub>               |
|    | (P31      |   |
| 25 | fragment) |   |
|    | ZElan040  | H <sub>2</sub> N-K (dns) SNPRGRRHPG-CONH <sub>2</sub>               |
|    | (P31      |   |
|    | fragment) |   |
|    | ZElan041  | H <sub>2</sub> N-K (dns) ENANT-CONH <sub>2</sub>                    |
|    | (P31      |   |
|    | fragment) |   |
| 30 | ZElan042  | H <sub>2</sub> N-K (dns) ANTRKS-CONH <sub>2</sub>                   |
|    | (P31      |   |
|    | fragment) |   |
|    | ZElan043  | H <sub>2</sub> N-K (dns) TRKSS-CONH <sub>2</sub>                    |
|    | (P31      |   |
|    | fragment) |   |
|    | ZElan044  | H <sub>2</sub> N-K (dns) RKSSR-CONH <sub>2</sub>                    |
|    | (P31      |   |
| 35 | fragment) |   |
|    | ZElan045  | H <sub>2</sub> N-K (dns) KSSRSN-CONH <sub>2</sub>                   |
|    | (P31      |   |
|    | fragment) |   |



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|                                 |   |
|---------------------------------|---|
| ZElan046<br>(P31<br>fragment)   | H <sub>2</sub> N-K (dns) SSRSNPG-CONH <sub>2</sub>                    |
| ZElan047<br>(P31<br>fragment)   | H <sub>2</sub> N-K (dns) RSNPRG-CONH <sub>2</sub>                     |
| ZElan048<br>(P31<br>fragment)   | H <sub>2</sub> N-K (dns) SNPRG-CONH <sub>2</sub>                      |
| ZElan049<br>(P31<br>fragment)   | H <sub>2</sub> N-K (dns) PRGRRH-CONH <sub>2</sub>                     |
| ZElan050<br>(P31<br>fragment)   | H <sub>2</sub> N-K (dns) RRHPG-CONH <sub>2</sub>                      |
| ZElan051<br>(HepC)              | H <sub>2</sub> N-K (dns) KSSRGN-CONH <sub>2</sub>                     |
| ZElan052<br>(HepC)              | H <sub>2</sub> N-K (dns) KTSERSQPRGRRQPG-CONH <sub>2</sub>            |
| ZElan053<br>(P31<br>analog)     | H <sub>2</sub> N-K (dns) TrKSSrSNPrGrrHPG-CONH <sub>2</sub>           |
| ZElan054<br>(P31<br>analog)     | H <sub>2</sub> N-K (dns) TRKSSrSNPRGrRHPG-CONH <sub>2</sub>           |
| ZElan055<br>(PAX2<br>fragment)  | H <sub>2</sub> N-K (dns) TNAKHSSHN-CONH <sub>2</sub>                  |
| ZElan056<br>(PAX2<br>fragment)  | H <sub>2</sub> N-K (dns) RRLRTRSRPN-CONH <sub>2</sub>                 |
| ZElan057<br>(PAX2<br>fragment)  | H <sub>2</sub> N-K (dns) RRLRTRSR-CONH <sub>2</sub>                   |
| ZElan058<br>(PAX2<br>fragment)  | H <sub>2</sub> N-K (dns) RRLRTR-CONH <sub>2</sub>                     |
| ZElan059<br>(PAX2<br>analog)    | H <sub>2</sub> N-K (dns) rrLrTrSrPN-CONH <sub>2</sub>                 |
| ZElan060<br>(HAX42<br>fragment) | H <sub>2</sub> N-K (dns) SDHALGTNLRSDNAKEPGDYNCCGNG-CONH <sub>2</sub> |
| ZElan061<br>(HAX42<br>fragment) | H <sub>2</sub> N-K (dns) GDYNCCGNGNSTGRKVFNRRRPSAIP-CONH <sub>2</sub> |
| ZElan062<br>(HAX42<br>fragment) | H <sub>2</sub> N-K (dns) SDHALGTNLRSDNAKEPG-CONH <sub>2</sub>         |
| ZElan063<br>(HAX42<br>fragment) | H <sub>2</sub> N-K (dns) GDYNCCGNGNSTG-CONH <sub>2</sub>              |
| ZElan064<br>(HAX42<br>fragment) | H <sub>2</sub> N-K (dns) RKVFNRRRPSAIP-CONH <sub>2</sub>              |

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|  |  |  |   |
|--|--|--|---|
|  |  | ZElan065<br>(HAX42<br>fragment)<br>ZElan066<br>(HAX42<br>fragment)<br>ZElan067<br>(HAX42<br>fragment)<br>Elan018<br>(PAX2 no<br>dns)<br>Elan021<br>(HAX42 no<br>dns)<br>ZElan070<br>(HAX42<br>fragment)<br>ZElan071<br>(HAX42<br>fragment)<br>ZElan072<br>(HAX42<br>fragment)<br>ZElan073<br>(PAX2<br>fragment)<br>ZElan074<br>(PAX2<br>fragment)<br>ZElan075<br>(PAX2<br>fragment)<br>ZElan076<br>(PAX2<br>fragment)<br>ZElan077<br>(PAX2<br>fragment)<br>ZElan078<br>(PAX2<br>fragment)<br>ZElan079<br>(PAX2<br>fragment)<br>ZElan080<br>(PAX2<br>fragment)<br>ZElan081<br>(PAX2<br>fragment)<br>ZElan082<br>(PAX2<br>fragment)<br>Elan035 | H <sub>2</sub> N-K (dns) RKVFNRRRRPS-CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) NRRRPSAIPT-CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) NRRRPS-CONH <sub>2</sub><br>H <sub>2</sub> N-<br>STPPSREAYSRPYSVSDSDSDTNAKHSSHNRRRLRTRSRPNG-<br>CONH <sub>2</sub><br>H <sub>2</sub> N-SDHALGTNLRSDNAKEPGDYNCCGNGNSTGRKVFNRRRRPS<br>AIPT-CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) SDHALGTNLRSDNAKEPGDYNCCGNGNST-<br>CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) NLRSDNAKEPGDYNCCGNGNSTGRKVFNR-<br>CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) PGDYNCCGNGNSTGRKVFNRRRPSAIPT-CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) ASHNRRRLRTR-CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) SAHNRRRLRTR-CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) SSANRRRLRTR-CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) SSHARRLRTR-CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) SSHNARLRTR-CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) SSHNRALRTR-CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) SSHNRRARTR-CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) SSHNRRLATR-CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) SSHNRRLRAR-CONH <sub>2</sub><br>H <sub>2</sub> N-K (dns) SSHNRRLRTA-CONH <sub>2</sub><br>H <sub>2</sub> N-SSHNRRRLRTR-CONH <sub>2</sub> |
|--|--|--|---|

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|    |          |                              |   |
|----|----------|------------------------------|---|
| 5  | ZElan113 | PAX2 Ala Scan 9              | H <sub>2</sub> N-K(dns) TNAKHSSHARLRTR        |
|    | ZElan114 | PAX2 Ala Scan 10             | H <sub>2</sub> N-K(dns) TNAKHSSHARLRTR        |
|    | ZElan115 | PAX2 Ala Scan 11             | H <sub>2</sub> N-K(dns) TNAKHSSHNRALRTR       |
|    | ZElan116 | PAX2 Ala Scan 12             | H <sub>2</sub> N-K(dns) TNAKHSSHNRRLRTR       |
|    | ZElan117 | PAX2 Ala Scan 13             | H <sub>2</sub> N-K(dns) TNAKHSSHNRRLATR       |
|    | ZElan118 | PAX2 Ala Scan 14             | H <sub>2</sub> N-K(dns) TNAKHSSHNRRLRAR       |
|    | ZElan119 | PAX2 Ala Scan 15             | H <sub>2</sub> N-K(dns) TNAKHSSHNRRLRTA       |
|    | ZElan123 | PAX2 15 mer<br>cyclic D form | H <sub>2</sub> N-K(dns) Lys-TNAKHSSHNRrLrTr   |
| 10 | ZElan124 | PAX2 15 mer D<br>form        | H <sub>2</sub> N-K(dns) TNAKHSSHNRrLrTr       |
|    | ZElan125 | PAX2 10 mer<br>cyclic        | H <sub>2</sub> N-K(dns) Lys-SSHNRRLRTR<br>[ ] |
|    | ZElan126 | PAX2 10 mer<br>cyclic D form | H <sub>2</sub> N-K(dns) Lys-SSHNRrLrTr<br>[ ] |
|    | ZElan127 | PAX2 10 mer<br>cyclic        | H <sub>2</sub> N-K(dns) Lys-TNAKHSSHNR<br>[ ] |
| 15 | ZElan128 | PAX2 10 mer<br>cyclic D form | H <sub>2</sub> N-K(dns) Lys-TNAKHSSHNR<br>[ ] |
|    | ZElan129 | PAX2 15 mer                  | H <sub>2</sub> N-K(dns) TNAKHSSHNRRLRTR       |
|    | ZElan130 | HAX42 14 mer Ala<br>Scan 1   | H <sub>2</sub> N-K(dns) AGDYNCCGNGNSTG        |
| 20 | ZElan131 | HAX42 14 mer Ala<br>Scan 2   | H <sub>2</sub> N-K(dns) PADYNCCGNGNSTG        |
|    | ZElan132 | HAX42 14 mer Ala<br>Scan 3   | H <sub>2</sub> N-K(dns) PGAYNCCGNGNSTG        |
|    | ZElan133 | HAX42 14 mer Ala<br>Scan 4   | H <sub>2</sub> N-K(dns) PGDANCCGNGNSTG        |
|    | ZElan134 | HAX42 14 mer Ala<br>Scan 5   | H <sub>2</sub> N-K(dns) PGDYACCGNGNSTG        |
| 25 | ZElan135 | HAX42 14 mer Ala<br>Scan 6   | H <sub>2</sub> N-K(dns) PGDYNACGNGNSTG        |
|    | ZElan136 | HAX42 14 mer Ala<br>Scan 7   | H <sub>2</sub> N-K(dns) PGDYNACGNGNSTG        |
|    | ZElan137 | HAX42 14 mer Ala<br>Scan 8   | H <sub>2</sub> N-K(dns) PGDYNCCANGNSTG        |
|    | ZElan138 | HAX42 14 mer Ala<br>Scan 9   | H <sub>2</sub> N-K(dns) PGDYNCCGAGNSTG        |
| 30 | ZElan139 | HAX42 14 mer Ala<br>Scan 10  | H <sub>2</sub> N-K(dns) PGDYNCCGNANSTG        |
|    | ZElan140 | HAX42 14 mer Ala<br>Scan 11  | H <sub>2</sub> N-K(dns) PGDYNCCGNGASTG        |
|    | ZElan141 | HAX42 14 mer Ala<br>Scan 12  | H <sub>2</sub> N-K(dns) PGDYNCCGNGNATG        |
| 35 | ZElan142 | HAX42 14 mer Ala<br>Scan 13  | H <sub>2</sub> N-K(dns) PGDYNCCGNGNSAG        |
|    | ZElan143 | HAX42 14 mer Ala<br>Scan 14  | H <sub>2</sub> N-K(dns) PGDYNCCGNGNSTA        |

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Table 21

| Source | Clone # | gst Fusion Sequence                           | SEQ ID NO. |
|--------|---------|---|------------|
| DCX11  | 98      | gst-SQSKQCMQYRTGRLTVGSEYCGMNPATPAYPARLLPRYR   | 213        |
| HAX42  | 99      | gst-SDHALGTNLRSDNAKEPGDYNCNGNSTGRKVFNRPRPSAIP | 214        |
| SNi34  | 100     | gst-SPCGGSGWGRFMQGLFGGRTDCCGAHRNRTSASLEPPSSDY | 215        |
| 5PAX5  | 97      | gst-RGSTGTAGGERSGVNLHTRDNASGSGFKWPYPSNRGKH    | 216        |
| SNi28  | 84      | gst-SHSGGMNRAYGDVRFELDRWNATSHHTRTPQLPRGPN     | 217        |
| SNi28  | 85      | gst-SHSGGMNRAY                                | 218        |
| SNi28  | 86      | gst-GDVRFELDR                                 | 219        |
| SNi28  | 87      | gst-WNATSHHTRP                                | 220        |
| SNi28  | 88      | gst-TPQLPRGPN                                 | 221        |
| SNi28  | 89      | gst-GDVRFELDRWNATSHHTRP                       | 222        |
| SNi28  | 90      | gst-WNATSHHTRTPQLPRGPN                        | 223        |
| SNi28  | 91      | gst-GDVRFELDRWNATSHHTRTPQLPRGPN               | 224        |
| SNi28  | 92      | gst-SHSGGMNRAYGDVRFELDRWNATSAATRTPQLPRGPN     | 225        |
| P31    | 93      | gst-SARDSGPAEDGSRVRLNGVENANTRKSSRNPRGRHP      | 226        |
| P31    | 101     | gst-SARDSGPAEDGSRVRLNG                        | 227        |
| P31    | 102     | gst-DGSRAVRLNGVENANTRKSSR                     | 228        |
| P31    | 103     | gst-ENANTRKSSRNPRGRHP                         | 229        |
| P31    | 110     | gst-ENANTRKSSR                                | 230        |

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|       |     |   |  |     |
|-------|-----|---|--|-----|
| P31   | 111 | gst-RKSSRSNPRG                                  |  |     |
| P31   | 112 | gst-SNPRGRHP                                    |  | 232 |
| P31   | 119 | gst-TRKSSRSNPRG                                 |  | 233 |
| PAX2  | 94  | gst-STPPSREAYSRPYSVSDSDTNAKHSSHNRLRTRSRPN       |  | 234 |
| PAX2  | 104 | gst-STPPSREAYSRPYSVSDSDSD                       |  | 235 |
| PAX2  | 105 | gst-SRPYSVSDSDSDTNAKHSSHN                       |  | 236 |
| PAX2  | 106 | gst-TNAKHSSHNRLRTRSRPN                          |  | 237 |
| PAX2  | 113 | gst-TNAKHSSHN                                   |  | 238 |
| PAX2  | 114 | gst-SSHNRLRTR                                   |  | 239 |
| PAX2  | 115 | gst-RRRLRTRSRPN                                 |  | 240 |
| SNi10 | 96  | gst-RVGQCTSDVRRPWARSCAHQCGGAGTRNSHGCTRPLRQASAH  |  | 241 |
| SNi10 | 116 | gst-RVGQCTSDVRRPWARSCA                          |  | 242 |
| SNi10 | 117 | gst-VRRPWARSCAHQCGGAGTRNS                       |  | 243 |
| SNi10 | 118 | gst-GTRNSHGCTRPLRQASAH                          |  | 244 |
| DCX8  | 95  | gst-RYKHDIGCDAGVDKKSSSVRGCGGAHSSPPRAGRPRGTMVSRL |  | 245 |
| DCX8  | 107 | gst-RYKHDIGCDAGVDKKSSSVRGCGG                    |  | 246 |
| DCX8  | 108 | gst-GCDAGVDKKSSSVRGCGGAHSSPPRA                  |  | 247 |
| DCX8  | 109 | gst-GAHSSPPRAGRPRGTMVSRL                        |  | 248 |

6.10.6. Peptide Stability

The relative stability for ZElan031, ZElan053 and ZElan054 was determined in simulated intestinal fluid (SIF) SIF was made by dissolving 100mg of pancreatin (Sigma cat#P-1625, lot# 122H0812) in 8.4ml of phosphate stock solution, adjusting the pH to 7.5 with 0.2N NaOH and adjusting the volume to 10ml with water.

Peptide (3.25mg) was dissolved in 3.25 ml of 10,000 fold diluted SIF solution at 37°C. Aliquots (0.7ml) of the digestion solution were then withdrawn at <1min, 1h, 3h, and 21h or 24h. The samples were quickly passed through a syringe filter (Millipore Millex-GV 0.22µm, part# SLGV025LS, lot# H2BM95250) and 300µL of the filtered solution was immediately injected onto a Hewlett-Packard HPLC system equipped with a C-8 column (Applied Biosystems column and guard column: column- p/n 0711-0023 Spheri-5 ODS 5µm, 220x4.6mm). The products were eluted at 1.5ml/min using an acetonitrile-water gradient. The major fluorescent peaks were collected, lyophilized and identified by MS analysis.

The HPLC gradient used was:

| Time (min) | Solvent Mixture   |
|------------|---|
| 0          | 95% H <sub>2</sub> O-5% acetonitrile (0.1%TFA)                        |
| 5          | 95% H <sub>2</sub> O-5%acetonitrile (0.1%TFA)                         |
| 35         | 85% H <sub>2</sub> O-15% acetonitrile (0.1%TFA) linear solvent change |
| 40         | 0% H <sub>2</sub> O-100% acetonitrile (0.1%TFA) "                     |
| 45         | 95% H <sub>2</sub> O-5% acetonitrile (0.1%TFA) "                      |
| 52         | 95% H <sub>2</sub> O-5%acetonitrile (0.1%TFA) "                       |

As shown in Table 22, the relative stability (to SIF) for the three peptides was found to be ZElan053>ZElan054>ZElan031. Enzymatic cleavage of the peptide was found to occur at arginine and/or lysine as expected. The replacement of l-amino acids with their D-amino acid analogs significantly reduced the rate of proteolysis at these residues.

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TABLE 22

|   | Peptide  | Percent Remaining at: |      |      |      | Rel.<br>Stab. |
|---|----------|-----------------------|------|------|------|---------------|
|   |          | 1 m                   | 1 h  | 3 h  | 24 h |               |
| 5 | ZElan031 | 100                   | 38.7 | 0    | 0    | 3             |
|   | ZElan054 | 97.4                  | 58.2 | 11.6 | 2.7  | 2             |
|   | ZElan053 | 100                   | 98.3 | 98.1 | 94.0 | 1             |

# 10 7. CHARACTERIZATION OF PEPTIDE-COATED PARTICLES

## Binding of Peptide-Coated PLGA Nanoparticles to Fixed Caco-2 Cells

Binding of nanoparticles coated with targeting peptides to fixed Caco-2 cells was investigated using an ELISA assay based on reaction of antibody with the dansyl moiety present on the peptides. Isoelectric points of selected synthetic peptides are shown in Table 23 (corresponding SEQ ID NOS. are shown in Table 7). Corresponding dansylated synthetic GIT binding peptides are given in Table 24.

TABLE 23

|    | Peptide | Sequence                                      | pI    |
|----|---------|---|-------|
|    | P31     | SARDSGPAEDGSRAVRLNGVENANTRKSSRSNPRGRRHP       | 12.26 |
| 25 | 5PAX5   | RGSTGTAGGERSGVLNLHTRDNASGSGFKPWYPSNRGHK       | 11.49 |
|    | SNi10   | RVGQCTDSDVRRPWARSCAHQCGAGTRNSHGCITRPLRQASAH   | 10.45 |
|    | SNi34   | SPCGGSWGRFMQGGFLFGGRTDGCGAHRNRTSASLEPPSSDY    | 8.25  |
|    | DCX11   | SQGSKQCMQYRTGRLTVGSEYGCGMNPARGHATPAYPARLLPRYR | 10.44 |
|    | DCX8    | RYKHDIGCDAGVDKKSSSVRGCGAHSSPPRAGRGRGTMTVSRL   | 11.03 |
|    | HAX42   | SDHALGTNLRSDNAKEPGDYNCCGNGNSTGRKVFNRRRPSAIP   | 9.62  |
| 30 | PAX2    | STPPSREAYSRPYSVSDSDTNAKHSSHNRRLRTRSRPN        | 11.26 |

TABLE 24

| <u>Peptide</u> | <u>Sequence</u>  |
|----------------|--|
| P31            | H <sub>2</sub> N-K(dns)SARDSGPAEDGSRVRLNGVENANTRKSSRSNPRGRRHPGG-CONH <sub>2</sub>      |
| 5PAX5          | H <sub>2</sub> N-K(dns)RGSTGTAGGERSGVLNLHTRDNASGSGFKPWYPSNRGHK-CONH <sub>2</sub>       |
| 5SNI10         | H <sub>2</sub> N-K(dns)RVGQCTDSDVRRPWARSACAHQCGAGTRNSHGCITRPLRQASAH-CONH <sub>2</sub>  |
| 5SNI34         | H <sub>2</sub> N-K(dns)SPCGGSWGRFMQGGFLGGRTDGC GAHRNRTSASLEPPSSDY-CONH <sub>2</sub>    |
| DCX11          | H <sub>2</sub> N-K(dns)SQGSKQCMQYRTGRLTVGSEYGC GMNPARHATPAYPARLLPRYR-CONH <sub>2</sub> |
| DCX8           | H <sub>2</sub> N-K(dns)RYKHDIGCDAGVDKSSSVRGCGAHSSPPRAGRGRGTMTVSRL-CONH <sub>2</sub>    |
| HAX42          | H <sub>2</sub> N-K(dns)SDHALGTNLRSDNAKEPGDYNCCGNGNSTGRKVFNRRRPSAIPT-CONH <sub>2</sub>  |
| PAX2           | H <sub>2</sub> N-K(dns)STPPSREAYSRPYSVSDSDTNAKHSSHNRLRTRSRPNG-CONH <sub>2</sub>        |
| 10DAB10        | H <sub>2</sub> N-K(dns)SKSGEGGDSSRGETGWARVRSHAMTAGRFRWYNQLPSDR-CONH <sub>2</sub>       |

**Method:**

Confluent Caco-2 monolayers grown in 96-well plates (p38) were fixed and treated with 0.1% phenylhydrazine before blocking with 0.1% BSA in PBS. Control and dansyl peptide-coated nanoparticles were resuspended in sterile water at 10mg/ml and stirred with a magnet for 1h at room temperature. Samples consisted of: (1) blank nanoparticle control, (2) scrambled PAX2-coated nanoparticles, (3) PAX2-coated nanoparticles, (4) HAX42-coated nanoparticles, (5) PAX2/HAX42-coated nanoparticles, and (6) 8 peptide-coated nanoparticles.

Nanoparticles were added to the cells at 10mg/ml in 100μl 1%BSA-PBS (no Tween80 is used in this assay) and 2-fold serially-diluted. The 96-well plates were incubated for 1h at room temperature. The plates were washed 5 times with 1%BSA-PBS and 100μl of anti-dansyl antibody (Cytogen DB3-226.3; 0.5 μg/ml; batch May 1997) was added per well and the plates incubated 1h at room temperature. The wells were washed 5 times with 1%BSA-PBS; 100μl of goat anti-mouse λ:HRP antibody (Southern Biotechnology CN. 1060-05; 1:10,000) was added per well, and the plates incubated 1h at room temperature. After washing 5 times with 1%BSA-PBS, 100μl of TMB peroxidase substrate (KPL CN. 50-76-00) was added to the wells and the optical density at 650nm was measured after 15 minutes.

As shown in Figures 13A-B, a decreasing anti-dansyl ELISA response was observed for nanoparticles coated with PAX2, HAX2, PAX2+HAX2, and a mixture of 8 targeting peptides, when decreasing amounts of the nanoparticles were applied to 5 fixed Caco-2 cells. No concentration effect was observed for blank nanoparticles or nanoparticles coated with a scrambled version of PAX2 peptide. Nanoparticles coated with PAX2, HAX2, PAX2+HAX2, and the 8 peptide mix, showed increased response relative to blank nanoparticles or nanoparticles 10 coated with a scrambled version of PAX2 peptide. The OD values were low relative to those normally observed for GST-peptide fusion binding to fixed Caco-2 cells.

Table 25 below shows the insulin potency and level 15 of peptides coated onto the particles (measured by fluorescence) for formulation 1 particles (formulation by the coacervation method given below).

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**Table 25**

| Peptide           | Blend           |                  |
|-------------------|-----------------|------------------|
|                   | Insulin<br>mg/g | Peptide<br>μl/mg |
| PAX2              | 60.7            | 3.51             |
| HAX42             | 55.9            | 2.93             |
| 25 PAX2 SCRAMBLED | 57.7            | 1.26             |
| P31               | 67.0            | 1.22             |
| 5PAX5             | 52.7            | 2.83             |
| SNi10             | 59.5            | 1.75             |
| SNi34             | 61.5            | 4.03             |
| DCX8              | 59.1            | 1.87             |
| DAB10             | 55.9            | 1.99             |

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### **ELISA of dansylated peptides and insulin coated PLGA particles**

The standard ELISA procedure was modified as 35 follows. Peptides and particles were diluted to an appropriate concentration in PBS containing 1%BSA (particles were sonicated to achieve a homogeneous solution), titered

T, 1080

and incubated one hour at room temperature. Following five washes with PBS containing 1%BSA, an in-house IgG1 $\lambda$  anti-dansyl monoclonal antibody was added (diluted to 1 $\mu$ g/ml in 1%BSA-PBS) and the plates were incubated for one hour. After 5 five more washes goat anti-mouse  $\lambda$ -HRP was added (Southern Biotechnology Associates Inc., Birmingham, AL, diluted 1:10,000 in 1%BSA-PBS) and the plates were incubated one hour. After five washes, plates were developed with TMB peroxidase substrate (Kirkegard and Perry, Gaithersburg, MD). 10 All data is presented with background binding subtracted. Tween 20 was not added to the diluent or the washes when insulin coated PLGA particles were included in the assay.

Figures 14A-14B show the binding of the dansylated 15 peptide SNI10 to hSI and BSA.

8. **BINDING OF SYNTHETIC PEPTIDES AND PEPTIDE-COATED PARTICLES TO S100 AND P100 FRACTIONS DERIVED FROM CACO-2 CELLS**

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8.1. **Detection of Binding to Membrane (P100) and Cytosolic (S100) fractions**

Caco-2 cell membrane (P100) and cytosolic (S100) fractions were prepared using a modification of the method described in Kinsella, B. T., O'Mahony, D. J. and G. A. 25 FitzGerald, 1994, J. Biol. Chem. 269(47): 29914-29919. Confluent Caco-2 cell monolayers (grown in 75 cm<sup>2</sup> flasks for up to 1 week at 37°C and 5% CO<sub>2</sub>) were washed twice in Dulbecco's PBS (DPBS) and the cells were harvested by 30 centrifugation at 1000 rpm after treatment with 10 mM EDTA-DPBS. The cells were washed 3 times in DPBS and the final cell pellet was resuspended in 3 volumes of ice cold HED buffer (20 mM HEPES (pH 7.67), 1 mM EGTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride (PMSF)). 35 The cells were allowed to swell for 5 min on ice prior to homogenization for 30 sec. The homogenates were centrifuged at 40,000 rpm for 45 min at 4°C. The supernatant (S100) was

removed and the pellet (P100) was resuspended in HEDG buffer (20 mM HEPES (pH 7.67), 1 mM EGTA, 0.5 mM dithiothreitol, 100 mM NaCl, 10% glycerol, 1 mM PMSF). Protein concentrations were determined using the Bradford assay (Bradford, M. M., 5 1976, Anal. Biochem. 72: 248-254).

Binding of peptide and/or peptide-coated PLGA particles to membrane (P100) and cytosolic (S100) fractions was assessed by detection of the dansyl moiety incorporated in the peptide. Costar ninety six well ELISA plates were 10 coated with S100 and P100 fractions (100 µg/ml in 0.05 M NaHCO<sub>3</sub>) overnight at 4°C. The plates were blocked with 0.5% bovine serum albumin in DPBS for 1 h at room temperature and washed 3 times in 1% BSA-DPBS. Peptide-coated particles or 15 peptides were dispersed in the same buffer and added to the plates at concentrations in the range 0.0325 - 0.5 mg/well. After 1 h at room temperature the plates were washed 5 times in 1% BSA-DPBS and 100 µl of anti-dansyl antibody (Cytogen DB3-226.3; 0.5 µg/ml) was added per well. The plates were incubated for 1 h at room temperature. The wells were washed 20 3 times in 1% BSA-DPBS and 100 µl of goat anti-mouse IgGλ:HRP antibody (Southern Biotechnology 1060-05; 1:10,000) was added per well. The plates were incubated for 1 h at room temperature. After washing 3 times in 1% BSA-DPBS 100 µl of TMB substrate (3,3',5',5'-tetramethylbenzidine; Microwell 25 Peroxidase Substrate System (Kirkegaard and Perry Laboratories 50-76-00)) was added and the optical density was measured at 650 nm at various time intervals.

### 8.2. Binding of Peptide-Coated PLGA particles

30 A novel assay system is provided by the instant invention for detection of binding of peptide-coated PLGA particles to membrane (P100) and cytosolic (S100) fractions derived from live Caco-2 cells. The absorbance readings obtained using this assay system were substantially higher 35 than those obtained using similar peptide-coated PLGA particle concentrations on fixed Caco-2 cells. This greater sensitivity together with the derivation of the S100 and P100

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were low and binding of the PAX2 (5 mg/g) was not consistent with this pattern.

Using particle concentrations of 0.03125 - 0.0625 mg/well all test peptide coated particles except PAX2 (0.05 mg/g) exhibited comparable or greater binding to both the S100 and P100 fractions than the scrambled PAX2 coated control particles. All particles exhibited greater binding to the P100 fraction than the S100 fraction. Binding to both the S100 and P100 fractions was directly proportional to the concentration of the PAX2 peptide on the particle. The absorbance readings obtained using this assay system were substantially higher than those obtained on the fixed Caco-2 cells.

The effect of blocking solution on binding of peptide-coated PLGA particles to P100 fractions (Caco-2 P35) was assessed using 1% bovine serum albumin (BSA) and 1% milk powder blocking solutions to assess background binding. The following particles were assayed at concentrations in the range 0.03125 - 0.0625 mg/well: no peptide; scrambled PAX2; and a range of PAX2 coated particles having peptide concentrations from 5-0.05 mg/g. As previously observed using 1% BSA, all test peptide coated particles except PAX2 coated at 0.05 mg/g exhibited comparable or greater binding to the P100 fractions than the scrambled PAX2 coated control particles. Binding to P100 fractions was directly proportional to the concentration of the PAX2 peptide on the particle (although in this instance PAX2 (5 mg/g) exhibited slightly lower binding than PAX2 (1 mg/g)). A similar trend was observed using 1% milk powder and a particle concentration of 0.0625 mg/well. However all absorbance readings were low when 1% milk powder was used and the binding pattern was not detectable using particles at a concentration of 0.0625 mg/well.

Non-specific binding of peptide-coated PLGA particles to plastic was also assessed using 1% BSA and 1% milk powder blocking solutions. The binding pattern observed above could be detected when BSA was used; however, absorbance readings

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were substantially lower and binding of particles PAX2 (0.1 and 0.05 mg/g respectively) was not detectable. When 1% milk powder was used, all absorbance readings were low and no binding pattern was detectable. BSA was chosen for blocking 5 in subsequent assays.

### 8.3. Comparison of Peptide-Coated Particle and Synthetic Peptide Binding to P100 fractions

Binding of dansylated peptides to P100 fractions 10 was assessed to determine if peptide binding was predictive of peptide-coated particle binding. Figure 16 illustrates the data obtained for the dansylated peptides A) HAX42, P31 D-form and scrambled PAX2 and B) PAX2, HAX42 and scrambled PAX2.

Two consecutive assays produced substantial variations 15 in absorbance readings. Initially, the HAX42 peptide exhibited strong binding when compared to the scrambled PAX2 control. The P31 D-form peptide (ZElan053) exhibited binding at the highest dilution only. In the repeat assay, HAX42 also exhibited significant binding compared to the scrambled 20 PAX2 control. However, the scrambled PAX2 control and HAX42 produced relatively high absorbance values compared to those obtained in the previous assay. The PAX2 peptide was indistinguishable from the scrambled PAX2 control.

Peptide/particle binding correlation is summarized as follows 25 in Table 26:

TABLE 26

#### Peptide/particle assay correlation

| 30 | Peptide                               | Assay correlation |
|----|---------------------------------------|-------------------|
|    | HAX42                                 | +                 |
|    | PAX2                                  | +/-               |
|    | P31 D-form                            | -                 |
|    | Scrambled PAX2                        | +/-               |
|    | + positive; +/- equivocal; - negative |                   |

35 Peptide/particle binding correlated well for the HAX42 peptide. In contrast, no correlation could be detected

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for the P31 D-form (ZElan053) peptide. Since the P31 D-form peptide-coated particles exhibited greater binding to the S100 fraction than the P100 fraction (unlike the other test peptides) it appears that the particle binding interaction was non-specific or that some other molecule was competing for binding to the P100 fraction but not to the S100 fraction. Thus the peptide/particle assay correlation may be useful for distinguishing between specific and non-specific binding interactions. The scrambled PAX2 control produced variable results so that it was difficult to assess the PAX2 binding correlation.

#### 8.4. Determination of HAX42 and PAX2 Binding Motif Sequences

Peptides and GST fusion proteins of HAX42, PAX2 and various derivatives were assayed using peptide ELISA to P100 membrane fractions derived from Caco-2 cells. The GST-PAX2 protein and PAX2 peptide data indicate that a core binding motif lies in the amino acid sequence TNAKHSSHNRLRTR (SEQ ID NO:237) otherwise named GST-106 and ZElan033. Similarly, the HAX42 peptide data suggest that a core binding motif for HAX42 lies in the amino acid sequence PGDYNCCGNCNSTG (SEQ ID NO:62), otherwise named ZElan091.

The peptides and proteins were analyzed by a dansylated peptide ELISA method in which 96 well plates were coated overnight at 4°C with 100µl/well coating protein (normally 100µg/ml P100 membrane fraction) in 0.05M carbonate buffer pH9.6. Nonspecific binding was blocked using 200µl/well, 2% Marvel/PBS for 2 hours at 37°C prior to incubation with dansylated peptides. The plates were washed three times with PBS/0.05% Tween 20 and after each subsequent incubation step. The peptides were diluted in blocking solution at a starting concentration of 100µg/ml and diluted 1:2 downwards, 100µl/well, followed by incubation at room temperature for 1 hour, exactly. A buffer blank control was included to ensure that background binding to plastic was not due to the antibodies used in the assay system. To detect the

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dansylated peptides, a mouse anti-dansyl antibody (DB3, Cytogen Corp.) at 1:1340 dilution in blocking buffer and 100 $\mu$ l/well was added followed by incubation at room temperature for 1 hour. The plates were then incubated with an anti-mouse  $\lambda$ -HRP conjugated antibody (Southern Biotech 1060-05) at a 1:10,000 dilution in blocking solution, 100 $\mu$ l/well for 1 hour at room temperature. Plates were developed using 75 $\mu$ l/well Bionostics TMB substrate and incubated for approximately 10 minutes. The developing reaction was stopped using Bionostics Red Stop solution (25 $\mu$ l/well), and the optical density of the plates was read at 650nm.

GST-PAX2 Peptides - Relative Binding to P100 Fractions

After subtraction of the GST-peptide binding to plastic from P100 binding values, the binding of GST-PAX2 peptides were represented as a ratio of GST-HAX42 binding to P100, which was given the arbitrary value of 1.00. The following ratios were determined from binding to P100 of GST-peptides at a peptide concentration of 20 $\mu$ g/ml. Bold denotes positive binding to the P100 membrane fraction.

Table 27

|    | GST-peptide      | Value       |
|----|------------------|-------------|
| 25 | <b>GST-HAX42</b> | <b>1.00</b> |
|    | <b>GST-PAX2</b>  | <b>1.79</b> |
|    | GST-104          | 0.01        |
|    | GST-105          | -0.08       |
|    | <b>GST-106</b>   | <b>2.71</b> |
|    | GST-113          | 0.26        |
|    | GST-114          | 0.17        |
|    | GST-115          | 0.36        |
| 30 | GST              | 0.48        |

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Table 28

|                  | GST-peptide Amino Acid Sequence                |
|------------------|--|
| <b>GST-PAX2</b>  | <b>STPPSREAYSRPYSVDSDDTNAKHSSHNRRRLRTRSRPN</b> |
| GST-104          | STPPSREAYSRPYSVDSDD                            |
| GST-105          | STPPSREAYSRPYSVDSDDTNAKHSSHN                   |
| <b>5 GST-106</b> | <b>TNAKHSSHNRRRLRTRSRPN</b>                    |
| GST-113          | TNAKHSSHN                                      |
| GST-114          | SSHNRRRLRTRSRPN                                |
| GST-115          | RRLRTRSRPN                                     |

**PAX2 Peptides - Relative Binding to P100 Fractions**

10 ZElan021, full length HAX42, was given the arbitrary value of 1.00 for binding to P100 at a given peptide concentration determined from the signal-to-noise ratio data. PAX2 and its derivatives are given as a ratio of HAX42 value to reflect their binding abilities to P100 membrane fractions. 15 derived from a Caco-2 cell line as shown in Table 29. Table 30 provides a line-up of the PAX2 peptides showing the positive binding peptides in boldface. The GST-PAX2 peptide and PAX2 peptide data agree, demonstrating that a binding motif is in the amino acid sequence TNAKHSSHNRRRLRTR (GST-106 20 and ZElan033).

25

30

35

TABLE 29

|    | PAX2<br>peptide | Binding<br>value<br>at<br>20µg/ml | Binding<br>value<br>at<br>20µg/ml | Binding<br>value<br>at<br>50µg/ml | Binding<br>value<br>at<br>50µg/ml | Binding<br>value<br>at<br>50µg/ml<br>(Jackson<br>Ab) | Binding<br>value<br>at<br>50µg/ml<br>(Southern<br>Ab) |
|----|-----------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|--|---|
| 5  |                 |                                   |                                   |                                   |                                   |  |   |
|    | ZElan018        | -0.33                             | 1.07                              | 0.95                              | 1.01                              |  |   |
|    | ZElan032        | 1.43                              | 2.87                              | 0.95                              | 1.06                              |  |   |
|    | ZElan033        | 0.35                              | 1.57                              | 0.80                              | 0.66                              |  |   |
|    | ZElan035        | 0.12                              | 0.43                              | 0.81                              | 0.77                              |  |   |
|    | ZElan055        | 0.99                              | 0.73                              | 1.10                              | 0.59                              |  |   |
|    | ZElan056        | 0.00                              | 0.16                              | 0.21                              | 0.21                              |  |   |
|    | ZElan057        | 0.08                              |                                   | 0.56                              | 0.25                              |  |   |
| 10 | ZElan058        | 0.05                              |                                   | 0.47                              | 0.16                              |  |   |
|    | ZElan073        | 0.07                              |                                   | -0.11                             | 0.49                              | 0.66   | 0.49  |
|    | ZElan074        | 0.06                              |                                   | 0.82                              | 0.52                              | 0.71   | 0.48  |
|    | ZElan075        | 0.13                              |                                   | 0.52                              | 0.38                              | 0.47   | 0.32  |
|    | ZElan076        | 0.08                              |                                   | 1.00                              | 0.41                              | 0.60   | 0.42  |
|    | ZElan077        | 0.20                              |                                   | 0.76                              | 0.54                              | 0.73   | 0.52  |
|    | ZElan078        | 0.11                              |                                   | 0.87                              | 0.69                              | 0.68   | 0.47  |
|    | ZElan079        | 0.31                              |                                   | 0.97                              | 0.68                              | 0.83   | 0.53  |
|    | ZElan080        | 0.23                              |                                   | 0.84                              | 0.45                              | 0.67   | 0.38  |
| 15 | ZElan081        | 0.01                              |                                   | 0.89                              | 0.47                              |  |   |
|    | ZElan082        | 0.00                              |                                   | 0.92                              | 0.40                              |  |   |
|    | ZElan083        | 0.43                              | 0.63                              | 1.03                              | 0.88                              |  |   |
|    | ZElan084        | 1.06                              | 0.93                              | 1.16                              | 0.77                              |  |   |
| 20 |                 |                                   |                                   |                                   |                                   |  |   |
| 25 |                 |                                   |                                   |                                   |                                   |  |   |
| 30 |                 |                                   |                                   |                                   |                                   |  |   |
| 35 |                 |                                   |                                   |                                   |                                   |  |   |

Table 30

|    | PAX2<br>Peptide          | Amino acid sequence   | SEQ ID<br>NO: |
|----|--------------------------|---|---------------|
|    | ZElan018                 | H <sub>2</sub> N-K(dns)STPPSREAYSRPYSDSDTNAKHSSHNRRRLRTRSRPNG -CONH <sub>2</sub>  |               |
|    | ZElan032                 | H <sub>2</sub> N-K(dns)TNAKHSSHNRRRLRTRSRPN-CONH <sub>2</sub>                     |               |
|    | ZElan033                 | H <sub>2</sub> N-K(dns)TNAKHSSHNRRRLRTR-CONH <sub>2</sub>                         |               |
| 5  | ZElan034                 | H <sub>2</sub> N-K(dns)SSHNRRRLRTRSRPN-CONH <sub>2</sub>                          |               |
|    | ZElan035                 | H <sub>2</sub> N-K(dns)SSHNRRRLRTR-CONH <sub>2</sub>                              |               |
|    | ZElan055                 | H <sub>2</sub> N-K(dns)TNAKHSSHN-CONH <sub>2</sub>                                |               |
|    | ZElan056                 | H <sub>2</sub> N-K(dns)RRLRTRSRPN-CONH <sub>2</sub>                               |               |
|    | ZElan057                 | H <sub>2</sub> N-K(dns)RRLRTRSR-CONH <sub>2</sub>                                 |               |
|    | ZElan058                 | H <sub>2</sub> N-K(dns)RRLRTR-CONH <sub>2</sub>                                   |               |
|    | ZElan059                 | H <sub>2</sub> N-K(dns)rrLrTrSrPN-CONH <sub>2</sub>                               |               |
|    | ZElan073                 | H <sub>2</sub> N-K(dns)ASHNRRRLRTR-CONH <sub>2</sub>                              |               |
| 10 | ZElan074                 | H <sub>2</sub> N-K(dns)SAHNRRRLRTR-CONH <sub>2</sub>                              |               |
|    | ZElan075                 | H <sub>2</sub> N-K(dns)SSANRRRLRTR-CONH <sub>2</sub>                              |               |
|    | ZElan076                 | H <sub>2</sub> N-K(dns)SSHARRLRTR-CONH <sub>2</sub>                               |               |
|    | ZElan077                 | H <sub>2</sub> N-K(dns)SSHNARLRTR-CONH <sub>2</sub>                               |               |
|    | ZElan078                 | H <sub>2</sub> N-K(dns)SSHNRRALRTR-CONH <sub>2</sub>                              |               |
|    | ZElan079                 | H <sub>2</sub> N-K(dns)SSHNRRRARTR-CONH <sub>2</sub>                              |               |
|    | ZElan080                 | H <sub>2</sub> N-K(dns)SSHNRRLATR-CONH <sub>2</sub>                               |               |
|    | ZElan081                 | H <sub>2</sub> N-K(dns)SSHNRRRLRAR-CONH <sub>2</sub>                              |               |
|    | ZElan082                 | H <sub>2</sub> N-K(dns)SSHNRRRLRTA-CONH <sub>2</sub>                              |               |
|    | SCRAMBLED PAX2 PEPTIDES: |   |               |
| 15 | ZElan083                 | H <sub>2</sub> N-K(dns)GRNHDVVSSNTHKSYRSPRSASYPRLSNDRTDRTEPAPSS-CONH <sub>2</sub> |               |
|    | ZElan084                 | H <sub>2</sub> N-K(dns)RNTRNKTSRLSANPHRSR-CONH <sub>2</sub>                       |               |

#### HAX42 Peptides - Relative Binding to P100 Fractions

ZElan021, full length HAX42, was given the arbitrary value of 1.00 for binding to P100 at a given peptide concentration determined from the signal-to-noise ratio data. HAX42 and its derivatives are given as a ratio of HAX42 value to reflect their binding abilities to P100 membrane fractions derived from a Caco-2 cell line as shown in Table 31. Table 32 provides a line-up of the HAX42 peptides showing the positive binding peptides in boldface. A core binding motif appears to lie in the amino acid sequence PGDYNCCGNCNSTG (ZElan091).

**TABLE 31**

| HAX42 peptide | Binding value at 20µg/ml | Binding value at 50µg/ml | Binding value at 50µg/ml | Binding value at 25µg/ml | Binding value at 25µg/ml | Binding value at 25µg/ml |
|---------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| ZElan021      | 1.00                     | 1.00                     | 1.00                     | 1.00                     | 1.00                     | 1.00                     |
| ZElan060      | 0.44                     | 0.56                     | 0.43                     |                          |                          |                          |
| ZElan061      | 0.20                     | 0.60                     | 0.38                     |                          |                          |                          |
| 5 ZElan062    | 0.11                     | 0.42                     | 0.34                     |                          |                          |                          |
| ZElan065      | 0.00                     | 0.54                     | 0.30                     |                          |                          |                          |
| ZElan067      | 0.08                     | 0.52                     | 0.40                     |                          |                          |                          |
| ZElan070      | 0.59                     | 0.97                     | 0.39                     |                          |                          |                          |
| ZElan071      | 1.22                     | 0.89                     | 0.75                     |                          |                          |                          |
| ZElan072      | 0.83                     | 0.61                     | 0.88                     |                          |                          |                          |
| ZElan087      |                          |                          |                          | 0.46                     | 0.44                     |                          |
| ZElan088      |                          |                          |                          | 2.21                     | 1.41                     | 1.63                     |
| ZElan089      |                          |                          |                          | 0.55                     | 0.44                     | 0.49                     |
| 10 ZElan090   |                          |                          |                          | 2.06                     | 1.54                     | 2.16                     |
| ZElan091      |                          |                          |                          | 2.02                     | 1.37                     | 1.20                     |
| ZElan092      |                          |                          |                          | 1.41                     | 1.90                     | 0.91                     |
| ZElan093      |                          |                          |                          | 1.88                     | 1.37                     | 1.33                     |

**Table 32**  
Amino acid sequence

| HAX42 Peptide | Amino acid sequence   |
|---------------|---|
| 15 ZElan021   | H <sub>2</sub> N-K(dns)SDHALGTNLRSDNAKEPGDYNC CGNGNSTGRKVFNRRRPSA IPT-CONH <sub>2</sub> |
| ZElan060      | H <sub>2</sub> N-K(dns)SDHALGTNLRSDNAKEPGDYNC CGNG-CONH <sub>2</sub>                    |
| ZElan061      | H <sub>2</sub> N-K(dns)GNGNSTGRKVFNRRRPSA IPT-CONH <sub>2</sub>                         |
| ZElan062      | H <sub>2</sub> N-K(dns)SDHALGTNLRSDNAKEPG-CONH <sub>2</sub>                             |
| ZElan065      | H <sub>2</sub> N-K(dns)RKVFNRRRPS-CONH <sub>2</sub>                                     |
| ZElan067      | H <sub>2</sub> N-K(dns)NRRRPS-CONH <sub>2</sub>   |
| 20 ZElan070   | H <sub>2</sub> N-K(dns)SDHALGTNLRSDNAKEPGDYNC CGNGNST-CONH <sub>2</sub>                 |
| ZElan071      | H <sub>2</sub> N-K(dns)NLRSDNAKEPGDYNC CGNGNSTGRKVFNR-CONH <sub>2</sub>                 |
| ZElan072      | H <sub>2</sub> N-K(dns)PGDYNC CGNGNSTGRKVFNRRRPSA IPT-CONH <sub>2</sub>                 |
| ZElan087      | H <sub>2</sub> N-K(dns)SDHALGTNLRSDNAKEPGDY-CONH <sub>2</sub>                           |
| ZElan088      | H <sub>2</sub> N-K(dns)SDNAKEPGDYNC CGNGNSTG-CONH <sub>2</sub>                          |
| ZElan089      | H <sub>2</sub> N-K(dns)SDHALGTNLRSDNAK-CONH <sub>2</sub> -CONH <sub>2</sub>             |
| ZElan090      | H <sub>2</sub> N-K(dns)EPGDYNC CGNGNSTG   |
| ZElan091      | H <sub>2</sub> N-K(dns)PGDYNC CGNGNSTG-CONH <sub>2</sub>                                |
| ZElan092      | H <sub>2</sub> N-K(dns)PGDYNC CGNG-CONH <sub>2</sub>                                    |
| 25 ZElan093   | H <sub>2</sub> N-K(dns)NCCGNGNSTG-CONH <sub>2</sub>                                     |

## 9. FORMULATIONS

### General Method for Preparation of Coacervated Particles.

Solid particles containing a Therapeutic as defined herein are prepared using a coacervation method. The are particles are formed from a polymer and have a particle size of between about 10nm and 500 µm, most preferably 50 to 800 nm. In addition the particles contain targeting ligands which are incorporated into the particles using a number of methods.

The organic phase (B) polymer of the general method given above may be soluble, permeable, impermeable,

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biodegradable or gastroretentive. The polymer may consist of a mixture of polymer or copolymers and may be a natural or synthetic polymer. Representative biodegradable polymers include without limitation polyglycolides; polylactides; 5 poly(lactide-co-glycolides), including DL, L and D forms; copolyoxalates; polycaprolactone; polyesteramides; polyorthoesters; polyanhydrides; polyalkylcyanoacrylates; polyhydroxybutyrates; polyurethanes; albumin; casein; citosan derivatives; gelatin; acacia; celluloses; polysaccharides; 10 alginic acid; polypeptides; and the like, copolymers thereof, mixtures thereof and stereoisomers thereof. Representative synthetic polymers include alkyl celluloses; hydroxalkyl celluloses; cellulose ethers; cellulose esters; nitrocelluloses; polymers of acrylic and methacrylic acids 15 and esters thereof; dextrans; polyamides; polycarbonates; polyalkylenes; polyalkylene glycols; polyalkylene oxides; polyalkylene terephthalates; polyvinyl alcohols; polyvinyl ethers; polyvinyl esters; polyvinyl halides; polyvinylpyrrolidone; polysiloxanes and polyurethanes and co- 20 polymers thereof.

Typically, particles are formed using the following general method:

An aqueous solution (A) of a polymer, surface active agent, surface stabilising or modifying agent or salt, 25 or surfactant preferably a polyvinyl alcohol (PVA) or derivative with a % hydrolysis 50 - 100% and a molecular weight range 500 - 500,000, most preferably 80-100% hydrolysis and 10,000-150,000 molecular weight, is introduced into a vessel. The mixture (A) is stirred under low shear 30 conditions at 10- 2000 rpm, preferably 100-600 rpm. The pH and/or ionic strength of this solution may be modified using salts, buffers or other modifying agents. The viscosity of this solution may be modified using polymers, salts, or other viscosity enhancing or modifying agents.

35 A polymer, preferably poly(lactide-co-glycolide), polylactide, polyglycolide or a combination thereof or in any enantiomeric form or a covalent conjugate of the these

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polymers with a targeting ligand is dissolved in water miscible organic solvents to form organic phase (B). Most preferably, a combination of acetone and ethanol is used in a range of ratios from 0:100 acetone: ethanol to 100: 0

5 acetone: ethanol depending upon the polymer used.

Additional polymer(s), peptide(s) sugars, salts, natural/biological polymers or other agents may also be added to the organic phase (B) to modify the physical and chemical properties of the resultant particle product.

10 A drug or bioactive substance may be introduced into either the aqueous phase (A) or the organic phase (B). A targeting ligand may also be introduced into either the aqueous phase (A) or the organic phase (B) at this point.

The organic phase (B) is added into the stirred  
15 aqueous phase (A) at a continuous rate. The solvent is evaporated, preferably by a rise in temperature over ambient and/or the use of a vacuum pump. The particles are now present as a suspension (C). A targeting ligand may be introduced into the stirred suspension at this point.

20 A secondary layer of polymer(s), peptide(s) sugars, salts, natural/biological polymers or other agents may be deposited on to the pre-formed particulate core by any suitable method at this stage.

The particles (D) are then separated from the  
25 suspension (C) using standard colloidal separation techniques, preferably by centrifugation at high 'g' force, filtration, gel permeation chromatography, affinity chromatography or charge separation techniques. The supernatant is discarded and the particles (D) re-suspended  
30 in a washing solution (E) preferably water, salt solution, buffer or organic solvent(s). The particles (D) are separated from the washing liquid in a similar manner as previously described and re-washed, commonly twice. A targeting ligand may be dissolved in washing solution (E) at the final washing  
35 stage and may be used to wash the particles (D).

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The particles may then be dried. Particles may then be further processed for example, tabletted, encapsulated or spray dried.

The release profile of the particles formed above  
5 may be varied from immediate to controlled or delayed release dependent upon the formulation used and/or desired.

Drug loading may be in the range 0-90% w/w.

Targeting ligand loading may be in the range 0-90% w/w.

Specific examples include the following examples:

10

**EXAMPLE 1: Peptide added at the final washing stage**

**Product:** Bovine Insulin loaded nanoparticles

**Aim:** To prepare a 2g batch of insulin loaded  
nanoparticles at a theoretical loading of 50mg/g and with the  
15 peptide ZElan018 added.

**Formulation Details**

|                                  |                  |                             |
|----------------------------------|------------------|-----------------------------|
| RG504H                           | (Lot no. 250583) | 2.0g                        |
| Acetone                          |                  | 45ml                        |
| Ethanol:                         |                  | 5ml                         |
| 20 PVA (aq. 5%w/v)               |                  | 400ml                       |
| Bovine Insulin (Lot no. 86H0674) |                  | 100mg                       |
| Peptide: PAX2 (ZElan018)         |                  | 10mg/50ml dH <sub>2</sub> O |

**Experimental details:**

25 The 5% w/v PVA solution was prepared by heating water until near boiling point, adding PVA and stirring until cool. The organic phase was prepared by adding acetone, 45ml, and ethanol, 5ml, together. The polymer solution was prepared by adding RG504H, 2g, to the organic phase and  
30 stirring until dissolved. The IKA™ reactor vessel was set up, all seals greased and the temperature was set at 25°C. The PVA solution, 400ml, was added into the reactor vessel and stirred at 400 rpm.

Bovine insulin, 100mg, was added into the stirring PVA  
35 solution. Using clean tubing and a green needle, the polymer solution was slowly dripped in the stirring PVA solution with the peristaltic pump set at 40. The solvent was allowed to

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evaporate by opening the ports and allowing the dispersion to stir overnight at 400 rpm.

The suspension was centrifuged in a Beckman Ultracentrifuge™ with swing-out rotor at 12,500 rpm, 4°C. The 5 supernatant was decanted and discarded. The "cake" of particles was broken up and dH<sub>2</sub>O (200mls) was added to wash the particles. The centrifugation and washing steps were repeated twice.

The peptide solution, (ZElan018, 10mg in 50ml dH<sub>2</sub>O) 10 was prepared and added to the particles for a final washing stage. The suspended particles were centrifuged as before. The supernatant liquid was decanted, the 'cake' broken up, and the particles were dried in the vacuum oven.

The particles were ground, placed in a securitainer and 15 sent for analysis. The weight of particles recovered was 1.45g. A SEM showed discrete, reasonably spherical particles in the 300-500nm size range. The potency was 49.2mg/g (98.0% of label claim). Peptide loading was 2.42 µg/mg (48.4% of label claim).

20

**EXAMPLE 2: Peptide added at the beginning of manufacture**

**Product:** Bovine Insulin loaded nanoparticles

**Aim:** To prepare a 2g batch of insulin loaded nanoparticles at a theoretical loading of 50mg/g and with the 25 peptide ZElan018 added at the beginning of manufacture.

**Formulation Details**

|                   |                   |       |
|-------------------|-------------------|-------|
| RG504H            | (Lot no. 250583)  | 2.0g  |
| Acetone           |                   | 45ml  |
| Ethanol:          |                   | 5ml   |
| 30 PVA(aq. 5%w/v) |                   | 400ml |
| Bovine Insulin    | (Lot no. 65H0640) | 100mg |
| Peptide: PAX2     | (ZElan018ii)      | 10mg  |

**Experimental details:**

35 The 5% w/v PVA solution was prepared by heating water until near boiling point, adding PVA and stirring until cool. The organic phase was prepared by adding acetone,

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559750-07862060

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45ml, and ethanol, 5ml, together. The polymer solution was prepared by adding RG504H (polyactide-co-glycolide, Boehringer Ingelheim), 2g, to the organic phase prepared in step above and stirring until dissolved. The IKA™ reactor vessel was set up, all seals greased and the temperature was set at 25°C. The PVA solution, 400ml, was added into the reactor vessel and stirred at 400 rpm.

Bovine insulin, 100mg, was added into the stirring PVA solution. PAX2 (ZElan018ii, 10mg) was added to the stirring PVA solution. Using clean tubing and a green needle, the polymer solution was slowly dripped into the stirring PVA solution with the peristaltic pump set at 40. The solvent was allowed to evaporate by opening the ports and allowing the dispersion to stir overnight at 400 rpm. The suspension was centrifuged in a Beckman Ultracentrifuge™ with swing-out rotor at 12,500 rpm, 4°C. The supernatant was decanted and discarded.

The "cake" of particles was broken up and dH<sub>2</sub>O (200ml) was added to wash the particles. The centrifugation and washing steps were repeated twice. The 'cake' was broken up and the particles were dried in the vacuum oven.

The particles were ground, placed in a securitainer and sent for analysis. The weight of the particles recovered was 1.6g. The potency was 47.3mg/g (94.6% of label claim). Peptide loading was 1.689µg/mg (33.8% of label claim).

EXAMPLE 3 Peptide added 1 hour before centrifugation

**Product:** Bovine Insulin loaded nanoparticles

**Aim:** To prepare a 1g batch of insulin loaded nanoparticles at a theoretical loading of 50mg/g and with the peptide ZElan018 added 1 hour before centrifugation.

**Formulation Details**

|                                  |                  |        |
|----------------------------------|------------------|--------|
| RG504H                           | (Lot no. 250583) | 1.0g   |
| Acetone                          |                  | 22.5ml |
| Ethanol:                         |                  | 2.5ml  |
| PVA(aq. 5%w/v)                   |                  | 200ml  |
| Bovine Insulin (Lot no. 65H0640) |                  | 50mg   |

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0007949-054999

04611

Peptide: PAX2 (ZElan018)

5mg

**Experimental details:**

The 5% w/v PVA solution was prepared by heating  
5 water until near boiling point, adding PVA and stirring until  
cool. The organic phase was prepared by adding acetone,  
22.5ml, and ethanol, 2.5ml, together. The polymer solution  
was prepared by adding RG504H, 1g, to the organic phase  
prepared above and stirring until dissolved. The IKA™  
10 reactor vessel was set up, all seals greased and the  
temperature was set at 25°C. The PVA solution, 200ml, was  
added into the reactor vessel and stirred at 400 rpm.

Bovine insulin, 50mg, was added into the stirring  
PVA solution. Using clean tubing and a green needle, the  
15 polymer solution was slowly dripped in the stirring PVA  
solution with the peristaltic pump set at 40. The solvent  
was allowed to evaporate by opening the ports and allowing  
the dispersion to stir overnight at 400 rpm.

PAX2 (ZElan018 5mg) was added to the stirring  
20 particle suspension. After 1 hr, the suspension was  
centrifuged in a Beckman Ultracentrifuge™ with swing-out  
rotor at 12,500 rpm, 4°C. The supernatant was decanted and  
discarded. The "cake" of particles was broken up and dH<sub>2</sub>O  
(200ml) was added to wash the particles. The centrifugation  
25 and washing steps were repeated twice.

The 'cake' was broken up and the particles were  
dried in the vacuum oven. The particles were ground, placed  
in a securitainer and sent for analysis. Potency was  
20.75mg/g (41.5% of label claim). Peptide loading was  
30 1.256µg/mg (25.12 % of label claim).

**EXAMPLE 4: Leuprolide acetate loaded nanoparticles**

Aim: To prepare a 3g batch of leuprolide-acetate loaded  
nanoparticles at a theoretical loading of 20mg/g and with the  
35 peptide ZElan024 added.

**Formulation Details**

RG504H (Lot no. 271077)

3.0g

00079949-054599  
065750-07862060

05611

lab

|                                     |                             |
|-------------------------------------|-----------------------------|
| Acetone                             | 67.5ml                      |
| Ethanol:                            | 7.5ml                       |
| PVA(aq. 5%w/v)                      | 600ml                       |
| Leuprolide acetate (Lot no. V14094) | 60mg                        |
| 5 Peptide: P31 (ZElan024)           | 15mg/50ml dH <sub>2</sub> O |

#### Experimental details:

The PVA solution was prepared and the organic phase was prepared by adding acetone, 67.5ml, and ethanol, 7.5ml, together. The polymer solution was prepared by adding RG504H, 3g, to the organic phase prepared above and stirring until dissolved. The IKA™ reactor vessel was set up, all seals greased and the temperature was set at 25°C. The PVA solution, 600ml, was added into the reactor vessel and stirred at 400 rpm.

Leuprolide acetate, 60mg, was added into the stirring PVA solution. Using clean tubing and a green needle, the polymer solution, was slowly dripped in the stirring PVA solution with the peristaltic pump set at 40. The solvent was allowed to evaporate by opening the ports and allowing the dispersion to stir overnight at 400 rpm. The suspension was centrifuged in a Beckman Ultracentrifuge™ with swing-out rotor at 15,000 rpm, 4°C. The supernatant was decanted and retained for analysis.

The "cake" of particles was broken up and dH<sub>2</sub>O (200ml) was added to wash the particles. The centrifugation and washing steps were repeated twice.

The peptide solution (P31 (SEQ ID NO:43), 15mg in 50ml dH<sub>2</sub>O) was prepared and added to the particles for a final washing stage. The suspended particles were centrifuged as before. The supernatant liquid was decanted, and the particles were dried in the vacuum oven.

The particles were ground, placed in a securitainer and sent for analysis. The weight of particles recovered was 1.87g. SEM showed discrete, reasonably spherical particles in the 300-500nm size range. The potency was 4.7mg/g (23.4% of label claim). Peptide loading was 1.76µg/mg.

**EXAMPLE 5: Peptide added by 'spiking' polymer phase with polymer-peptide conjugate**

**Product:** Bovine Insulin loaded nanoparticles

**Aim:** To prepare a 3g batch of insulin loaded

5 nanoparticles at a theoretical loading of 50mg/g and with the polymer-peptide conjugate PLGA-ZElan019 added.

**Formulation Details**

RG504H (Lot no. 271077) 2.85g

RG504H-ZElan019 conjugate 0.15g

10 (5PAX5-conjugate)

Acetone 67.5ml

Ethanol: 7.5ml

PVA(aq. 5%w/v) 600ml

Bovine Insulin(Lot no. 86H0674) 150mg

15

**Experimental details:**

The 5% w/v PVA solution was prepared by heating water until near boiling point, adding PVA and stirring until cool. The organic phase was prepared by adding acetone, 20 67.5ml, and ethanol, 7.5ml, together. The polymer solution was prepared by adding RG504H and the polymer-peptide conjugate to the organic phase and stirring until dissolved.

The IKA™ reactor vessel was set up, all seals greased and the temperature was set at 25°C. The PVA 25 solution, 400ml, was added into the reactor vessel and stirred at 400 rpm.

Bovine insulin, 100mg, was added into the stirring PVA solution. Using clean tubing and a green needle, the polymer solution, was slowly dripped in the stirring PVA 30 solution with the peristaltic pump set at 40. The solvent was allowed to evaporate by opening the ports and allowing the dispersion to stir overnight at 400 rpm.

The suspension was centrifuged in a Beckman Ultracentrifuge™ with swing-out rotor at 12,500 rpm, 4°C. 35 The supernatant was decanted and discarded. The "cake" of particles was broken up and dH<sub>2</sub>O (200ml) was added to wash the

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particles. The centrifugation washing step was repeated twice.

The 'cake' was broken up and the particles were dried in the vacuum oven. The particles were ground, placed in a securitainer and sent for analysis. The weight of particles recovered was 2.8g. The potency was 53.1mg/g (106.2% of label claim). Peptide loading was 4.02 µg/mg (80.4% of label claim).

10. ANIMAL STUDIES

Study 1

An open-loop study in which the test solution was injected directly into the ileum was done. Wistar rats (300-350g) were fasted for 4 hours and anaesthetized by intramuscular administration 15 to 20 minutes prior to administration of the test solution with a solution of ketamine [0.525 ml of ketamine (100 mg/ml) and 0.875 ml of acepromazine maleate-BP ACP (2mg/ml)]. The rats were then injected with a test solution (injection volume: 1.5ml PBS) intra-duodenally at 2-3 cm below the pylorus. The test solution contained either PLGA particles manufactured according to the coacervation procedure given above with or without targeting peptides or by the "spiked" method given above. Insulin (fast-acting bovine; 28.1 iu/mg) was incorporated in the particles at 5% drug loading for a total of 100iu insulin (70 mg particles) or 300iu insulin (210 mg particles). Blood glucose values for the rats were measured using a Glucometer™ (Bayer; 0.1 to 33.3 m/mol/L); plasma insulin values were measured using a Phadeseph RIA Kit™ (Upjohn Pharmacia; 3 to 240 µU/ml-assayed in duplicate). Systemic and portal blood was sampled.

Study groups included animals receiving test solutions containing particles coated with the following peptides shown in Table 33.

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Table 33

|    |                    |                 |                      |
|----|--------------------|-----------------|----------------------|
|    | <b>Study Group</b> | <b>Receptor</b> | <b>Peptide</b>       |
|    | I                  | hSI             | SNi10                |
|    |                    |                 | SNi34                |
| 5  | II                 | hPEPT1          | P31                  |
|    |                    |                 | 5PAX5                |
|    | III                | HPT1            | PAX2                 |
|    |                    |                 | HAX42                |
|    | IV                 | D2H             | DCX8                 |
| 10 |                    |                 | DCX11                |
|    | V ("spiked")       | hPEPT1          | P31-PLGA conjugate   |
|    |                    |                 | 5PAX5-PLGA conjugate |

Control groups included: 1) PBS control (1.5ml) Open-Loop; 2) Insulin solution (1iu/0.2ml) subcutaneous; 3) Insulin particles - no peptide (1iu/0.2ml) subcutaneous; 4) Insulin particles/all 8 peptides mix (1iu/0.2ml) subcutaneous; 5) Insulin loaded particles/peptide control (scrambled 5PAX5) (100iu/1.5ml) Open-Loop; 6) Insulin loaded particles/peptide control (scrambled 5PAX5) (300iu/1.5ml) Open-Loop; 7) Control particles (insulin-free)/all 8 peptide mix (equivalent 100iu/1.5ml) Open-Loop; and 8) Control particles (insulin-free)/all 8 peptide mix (equivalent 300iu/1.5ml) Open-Loop.

The following describes the pharmacokinetics for 300iu-loading:

|    |                        |             |                        |                     |
|----|------------------------|-------------|------------------------|---------------------|
|    | <b>Target Receptor</b> | <b>F%**</b> | <b>Fold-increase**</b> | <b>Stat. Sig.**</b> |
|    | HPT1                   | 10.37       | 17.0                   | <0.001              |
|    | Spiked hPEPT1          | 4.94        | 7.5                    | 0.005               |
|    | PAX2 scrambled         | 3.50        | 3.6                    | NS                  |
|    | Mix-8                  | 2.00        | 2.0                    | NS                  |
| 30 | hPEPT1                 | 1.60        | 1.5                    | NS                  |
|    | D2H                    | 1.57        | 1.4                    | NS                  |
|    | hSI                    | 0.54        | 0.9                    | NS                  |

\* based on area under the curve (AUC) (1-4h), base-line adjusted, relative to subcutaneous insulin solution 1iu

\*\* Fold increase in AUC compared to insulin particles: 300iu

Figures 17A and 17B show the systemic blood glucose and insulin levels following intestinal administration of control (PBS); insulin solution; insulin particles; all 8



peptides mix particles and study group peptide-particles (100iu). Figures 18A and 18B show the systemic blood glucose and insulin levels following intestinal administration of control (PBS); insulin solution; insulin particles and study 5 group peptide-particles (300iu).

HPT1 targeted peptide coated particles provided the most potent enhancement of the delivery of insulin over subcutaneous injection of insulin followed by hPEPT1 spiked > PAX2 scrambled > mix-8 > hPEPT1 > D2H > uncoated particles > 10 hSI > solution. In a repeat study, the uncoated particles containing insulin gave similar profiles but the HPT1-peptide targeted particles gave a reduced profile (3-fold). The insulin-free PLGA particles and the all-8 mix particles did not show an effect on the basal insulin or glucose levels. 15 The HPT1 targeting particles, the PEPT1 spiked, targeting particles, and the PEPT1 targeting particles also reduced blood glucose levels indicative that the insulin delivered was bioactive. The other targeting particles were also shown to reduce blood glucose levels although not to the same 20 extent as the HPT1 and PEPT1 spiked particles. No histological differences were observed in the small intestine for any of the formulations evaluated.

### Study 2

25 A second open-loop study, similar to study 1 above, was undertaken with the following treatment groups as shown in Table 34.

Table 34

30

| Group Number | Dose Insulin (iu) | Description                  |
|--------------|-------------------|------------------------------|
| 1            |                   | PBS control                  |
| 2a           | 1                 | subcutaneous, bovine insulin |
| 2b           | 2                 | subcutaneous, bovine insulin |
| 2c           | 3                 | subcutaneous, bovine insulin |
| 2d           | 4                 | subcutaneous, bovine insulin |
| 2e           | 10                | subcutaneous, bovine insulin |

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|    |    |     |   |
|----|----|-----|---|
|    | 2f | 20  | subcutaneous, bovine insulin                        |
|    | 2g | 4   | subcutaneous, human insulin                         |
|    | 3  | 300 | uncoated insulin particles                          |
|    | 4  | 100 | HAX42/PAX2 with 300 iu particle loading             |
| 5  | 5  | 300 | HAX42/PAX2 (40mer) particles                        |
|    | 6  | 300 | HAX42 (40mer) particles                             |
|    | 7  | 300 | HAX42 particles + 10-fold excess free HAX42 (40mer) |
|    | 8  | 300 | PAX2 (40mer) particles                              |
|    | 9  | 300 | PAX2 freeze-dried (40mer) particles                 |
|    | 10 | 300 | PAX2 scrambled particles III (40mer)                |
| 10 | 11 | 300 | PAX2 scrambled particles IV (19mer)                 |
|    | 12 | 300 | 5PAX5/P31 (40mer) particles                         |
|    | 13 | 300 | P31 (40mer) particles                               |
|    | 14 | 300 | 5PAX5 (40mer) particles                             |
|    | 15 | 300 | HAX42 (27mer) particles                             |
| 15 | 16 | 300 | PAX2 (20mer) particles                              |
|    | 17 | 300 | P31 (20mer) particles                               |
|    | 18 | 300 | PAX2 (15mer) particles                              |
|    | 19 | 300 | P31 (15mer) particles                               |
|    | 20 | 300 | P31 D-form I(5 D-arginine) (16mer) particles        |
| 20 | 21 | 300 | P31 D-form II(2 D-arginine) (16mer) particles       |
|    | 22 | 300 | HAX42 (10mer)                                       |

Availability of insulin following administration was assessed relative to a 1 and 20iu subcutaneous dose because the response to increasing subcutaneous doses of bovine insulin does not increase linearly over the range of 1 to 20iu. Data up to three hours post-dosing was available for most animals. Therefore, availability was first assessed using individual AUC(0-3h) data estimated from baseline-subtracted data for which data up to 3 hours was available. This approach may lead to an underestimation of the availability as some animals that gave a high response often did not survive for 3 hours and, therefore, were excluded from the analyses. In an attempt to capture as much of these high responses observed at the earlier timepoints as possible, the mean baseline-subtracted plasma concentration

data was used to estimate an AUC for each group. Table 35 shows the results based on this second approach (AUC(0-3h) calculated from the mean plasma concentration data).

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Table 35

| Group    | Dose iu | Mean AUC <sub>(0-3h)</sub> | F vs. 1 iu | F vs. 20 iu |
|----------|---------|----------------------------|------------|-------------|
| 1        | 0       | 2.14                       |            |             |
| 2a       | 1       | 875.27                     | 100.00     | 28.86       |
| 2b       | 2       | 2439.36                    | 139.35     | 40.22       |
| 2c       | 3       | 3671.44                    | 139.82     | 40.36       |
| 2d       | 4       | 6912.18                    | 197.43     | 56.98       |
| 2e       | 10      | 27224.41                   | 311.04     | 89.77       |
| 2f       | 20      | 60651.28                   | 346.47     | 100.00      |
| 2g       | 4       | 14255.49                   | 407.17     | 117.52      |
| 3        | 300     | 10677.78                   | 4.07       | 1.17        |
| 3 -Rat43 | 300     | 4645.06                    | 1.77       | 0.51        |
| 4        | 100     | 3527.18                    | 4.03       | 1.16        |
| 5        | 300     | 27112.26                   | 10.33      | 2.98        |
| 6        | 300     | 33091.68                   | 12.60      | 3.64        |
| 7        | 300     | 9303.09                    | 3.54       | 1.02        |
| 8        | 300     | 34241.83                   | 13.04      | 3.76        |
| 9        | 300     | 10968.83                   | 4.18       | 1.21        |
| 10       | 300     | 27692.78                   | 10.55      | 3.04        |
| 11       | 300     | 3004.29                    | 1.14       | 0.33        |
| 12       | 300     | 18852.61                   | 7.18       | 2.07        |
| 13       | 300     | 20278.43                   | 7.72       | 2.23        |
| 14       | 300     | 17400.38                   | 6.63       | 1.91        |
| 15       | 300     | 16775.69                   | 6.39       | 1.84        |
| 16       | 300     | 14217.47                   | 5.41       | 1.56        |
| 17       | 300     | 8197.97                    | 3.12       | 0.90        |
| 18       | 300     | 25050.59                   | 9.54       | 2.75        |
| 19       | 300     | 7927.96                    | 3.02       | 0.87        |
| 20       | 300     | 21519.57                   | 8.20       | 2.37        |
| 21       | 300     | 6322.41                    | 2.41       | 0.69        |
| 22       | 300     | 12553.01                   | 4.78       | 1.38        |

The data for group 3 (uncoated insulin particles) are expressed with and without Rat 43. This animal had an atypically high response to these uncoated particles and, therefore, may have biased the data for this group.

This data shows that a combination of peptide-coated particles (HAX42/PAX2 or 5PAX5/P31) shows no greater availability than particles coated with the individual peptides. Further, peptide-coated particles have a greater availability than uncoated peptides. Scrambling the 40mer

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PAX2 peptide did not result in a loss of bioavailability. Scrambling the PAX2 peptide and reducing the size to 19mer resulted in a loss of bioavailability although this loss may be attributed in part to the reduction in peptide size.

- 5 Reducing peptide size resulted in loss of bioavailability. The D-form of P31 (ZElan053) had increased bioavailability possibly due to greater resistance to peptide breakdown. A competitive excess of peptide resulted in a loss of bioavailability, and freeze drying caused a loss in
- 10 bioavailability. By way of example, measurement of blood glucose levels showed that the HPT1 and hPEPT1 targeting particles incorporating HAX42, PAX2, P31 (SEQ ID NO:43), and P31 D-form (ZElan053) reduced blood glucose levels indicating that the insulin delivered was bioactive.
- 15 In further studies, insulin was recovered from the targeting particles following particle formation by dissolution and analyzed by electrophoresis in non-denaturing sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). The analysis of the insulin by non-
- 20 denaturing SDS-PAGE and also by western blot transferred to membranes and subsequent screening with an antibody to insulin, indicated that the insulin was intact, with no evidence of degradation, dimerization, or aggregation during the process of particle formation.

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### Study 3

- An intraduodenal open loop model study was carried out on Wistar rats (300-350g). Group 1 was administered leuprolide acetate (12.5  $\mu$ g) subcutaneously. Group 2 was
- 30 administered intraduodenally uncoated leuprolide acetate particles (600  $\mu$ g, 1.5 ml). Group 3 was intraduodenally administered leuprolide acetate particles coated with PAX2 (600  $\mu$ g; 1.5 ml). Group 4 was administered intraduodenally leuprolide acetate particles coated with P31 (SEQ ID NO:43)
- 35 (600  $\mu$ g, 1.5 ml). Figure 19 shows the leuprolide plasma concentration following administration to these four groups. Both the P31 (SEQ ID NO:43) and the PAX2 coated leuprolide

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particles administered intraduodenally provided enhanced plasma levels of leuprolide relative to subcutaneous injection.

- 5 Homologies of GIT transport-binding peptides to known proteins are shown in Figures 20, 21A-F, and 22 A-D.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed,  
10 various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

- 15 Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

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